REMARKS/ARGUMENTS

Claims 38, 40, 41, 46 and 48 are currently under examination.

Claim Rejections under 35 U.S.C. 112:

Claims 38, 40, 41, 46 and 48 are rejected under 35 U.S.C. 112, first paragraph, as allegedly lacking enablement. Claims 38, 40-41, 46, and 48 are further rejected under 35 U.S.C. 112, first paragraph as failing to comply with the written description requirement. Applicants respectfully traverse these rejections for the following reasons.

The Examiner maintains that the specification fails to provide sufficient information or guidance to use or conduct the recited method to achieve the claimed effect. The Examiner asserts that the technical data provided by the specification i.e., the results of the cytoadherence assays, are not adequate to reflect the effectiveness of an *in vivo* treatment for humans. The Examiner has cited Cooke et al. and Newbold et al. in support of his arguments and insists that more specific enablement is required in view of the uncertainty in the art disclosed by these citations.

In relation to the administration of L-arginine, as currently recited in claim 38, the Examiner asserts that the evidence submitted in the previous response does not demonstrate treatment of malaria by administering agents to increase nitric oxide levels. The Examiner asserts that the art is highly uncertain. This assertion is based on the Examiner's interpretation of conservative statements in Antsey et al., *Am. J. of Tropical Medicine and Hygiene*, 67(2): abst. 515, 2002. The Examiner contends that the method is possibly unreliable in view of the fact that no animal or human model (presumably clinical trials) systems have been tested.

Applicants do not agree. There are no mouse models of malaria in which NO production is suppressed. The present inventors provided the first evidence that NO production is reduced in human subjects with severe malaria. This discovery foreshadowed the present invention directed to administration of agents which increase

NO production as a treatment of malaria in humans. The mechanism by which NO reduces the pathology associated with severe malaria is proposed to be *via* reducing cytoadherence of parasitized erythrocytes to endothelial cells and specifically by reducing expression of adhesion molecules by endothelial cells. In severe malaria parasitized red blood cells cling to the endothelial lining of blood vessels and cause microvascular obstruction. Several such receptors are known, such as ICAM-1, VCAM-I and CD36.

As the Office Action shows, the Examiner has focused on the mechanism of action and whether or not the cytoadherence assay enables the claimed method. In response, Applicants emphasize the following aspects of the invention:

1. The present inventors have shown that high levels of NO in humans are associated with reduced pathology during malaria infection. Low levels of NO are associated with severe malaria with the lowest levels detected in subjects with very severe malaria.
2. Cytoadherence is an important measure of pathology of *Plasmodium* infection.
3. *In vitro* cytoadherence assays are the standard model in the field and correlate with pathology.
4. Increasing NO levels decreases cytoadherence and therefore decreases pathology irrespective of the particular endothelial receptor used.

In relation to cytoadherence assays and pathogenesis in malaria, Applicants further emphasize the following:

The cytoadherence assay is not so uncertain as asserted by the Examiner. There is no reliable rodent model for testing the role of cytoadherence by parasitized red cells in mediating pathogenesis *in vivo.* In contrast, measures of *in vitro* cytoadherence by parasitized red blood cells are recognized as correlating to pathogenicy. Thus, *in vitro* cytoadherence assays are the standard in the field. The overwhelming preponderance of evidence in severe and fatal malaria, including post mortem evidence, supports the conclusion that cytoadherence by parasitized red cells

to endothelial receptors, sequestration of parasitized red cells and microvascular obstruction is extremely important in the pathogenesis *of* malaria. See, for example, Ho and White, *Am. J. Physiol.*, 276: 1231, 1999 (submitted herewith as Exhibit A).

Regarding uncertainty in the art and the role *of* CD36 molecules in cytoadherence, Applicants submit that the Examiner's interpretation *of* Cooke el at. and Newbold et al. is not representative *of* the main stream scientific thought or *of* the autopsy evidence.

The Examiner appears to be arguing that if CD36 is not an important endothelial receptor mediating pathogenesis in malaria, then the present examples showing a reduction *of* cytoadherence to human C32 melanoma cells is somehow less valid. However, there is no indication that NO has a selective effect on CD36. Inhibition *of* parasite binding to CD36 on human melanoma cells is representative *of* the effect of NO on binding of parasitized red cells to cells and to endothelial adhesion molecules. Specifically, inhibition of binding by NO domains is a class effect irrespective of the relative importance of the representative molecule. Irrespective of the role of CD36, which is representative *of* adhesion molecules effected by NO, it is widely accepted that NO could inhibit cytoadherence through inhibition of endothelial adhesion molecules other than CD36, including ICAM-1 and VCAM-I. For example, De Caterina, *J. Clin. Invest*, 96(1):60-68, 1995 demonstrates the inhibitory effect *of* NO on endothelial expression of ICAM.I.

The criticisms perceived by the Examiner in Newbold et al. of the role of CD36 in pathogenesis are related to the fact that CD36 is not found on brain endothelium and therefore may not contribute to cerebral malaria. At the time the subject studies were performed, CD36 was considered an important molecule mediating binding and mediating pathogenesis of severe malaria and it remains so for non-cerebral severe malaria. There are leading authorities in the field who dispute the notion that CD36 is not important and who argue that CD36 extremely important in pathogenesis through synergy with other adhesions molecules (see enclosed review article by Ho and White).

CD36 is found in endothelial cells in other organ systems that are severely affected in severe malaria. Accordingly, the inhibition of CD36 data have therapeutic implications for a role of NO in ameliorating other end organ pathologies in organs such as renal failure, bone marrow failure, anaemia, and lung injury.

Applicants submit that the Examiner's reliance on the caution expression in Lopansri *et al.,* Lancet, 2003 regarding the role *of* arginine in therapeutics is not appropriate. Applicants argue that the data disclosed by Lopansri *el at.* would not generate this level of uncertainty for those skilled in the art. Such conservative expressions are standard in all medical journals and are often required by journal editors until a therapeutic role is proven beyond any doubt in at least one large randomized clinical trial. Applicants submit that at the priority date there was no uncertainty whether or not the administration of the proposed agents would have the effect specified i.e. an increase in NO. To illustrate this, submitted herewith are several scientific publications, published prior to the instant priority date showing increased NO production following administration *of* arginine either orally or intravenously: Bode-Boger S. M.. et al., *Cardiovasc. Res.* 37(3):756-764,1998 (Exhibit B); Schellong S. M., et al., *Clin. Sci. (Lond),* 93(2):159-165, 1997 (Exhibit C); Bode-Boger S. M., et al., *Circulation.* 93(1):85-90, 1996 (Exhibit D); and Bode-Boger S. M., et al., *Clin. Sci. (Lond),* 87(3);303-310, 1994 (Exhibit E). In relation to inhaled *NO,* Gries A. et al. *Circulation,* 97(15):1481-1487, 1998 (Exhibit F) showed increased NO levels after inhaled NO. For the skilled addressee at the priority date seeking to enhance NO levels in humans, the information in the fore-mentioned citations together with the description in the present specification would enable the invention. Anstey et al., *J. Exp. Med.* 184:557-567, 1996 describe how measurements of plasma and urine nitrate levels can demonstrate impairment of NO production and also quantify the magnitude of this impairment found in severe human malaria. Known amounts of NO and known amounts of oral/intravenous arginine increase NO production and urinary/plasma nitrate in humans and animals. The nitrate levels reported in malaria-exposed but healthy subjects would allow a reasonable calculation of the target level of NO production required.

In summary, Applicants maintain that the present disclosure combined with the knowledge available in the art at the filing date of the instant application does provide enablement for the claimed invention. Based on the above comments and arguments, Applicants further submit that the specification as filed does provide sufficient written description of the claimed invention to reasonably convey to those skilled in the art that the inventors had possession of the invention at the time this application was filed. The Examples in the specification are working examples for the purpose of supporting enablement and written description at the time of filing. Withdrawal of the rejection under 35 U.S.C. 112, first paragraph, is respectfully requested.

Claim Rejections under 35 U.S.C. 102:

Claims 38, 40, 46, and 48 are rejected under 35 U.S.C. 102(b) as allegedly anticipated by Rockett et al. Claims 38, 40, 46, and 48 are rejected under 35 U.S.C. 102(e) as allegedly anticipated by Stamler et al. Applicants respectfully traverse these rejections.

Claim 38 defines a method for inhibiting, retarding or killing of any life cycle stages of a *Plasmodium* species comprising administering to a host parasitized by said *Plasmodium* species an agent for a time and under conditions sufficient to inhibit or reduce pathologic adherence properties of the parasitized cells, wherein said agent is L-arginine, NO gas and/or an S-nitrosothiol compound.

In contrast to the Examiner's allegation, Rocket et al. does not teach the invention as claimed. The cited reference neither teaches nor suggests a protective role for NO in malaria. In fact, in all their writings from 1991 to 1998 the authors argue for a pathogenic (non protective) role of NO. When the authors of the cited reference address therapeutic implications, they argue for inhibition of NO rather than therapies to increase NO. Accordingly, it is submitted that the Examiner is incorrect in alleging that the citation anticipates the claimed invention.

With regard to Stamler et al., Applicants argue that the cited patent fails to teach a method of treating malaria by administering to a host an agent capable of increasing NO levels. The cited patent is broadly directed to loading of isolated red blood cells with reagents causing forming S-nitroslylation of thiol groups such as S-nitrosothiols. The loaded red blood cells are useful in methods of therapy for conditions characterized by abnormal O_2 metabolism such as sickle cell anemia, ischaemic injury, hypertension, etc.

The Examiner alleges that Stamler et al. teaches a method of treating the infected *Plasmodium falciparum* patient ex vivo by using the nitrothiol compounds. The invention claimed in the present case is not a method using ex vivo loading of isolated red blood cells to treat malaria. Regarding the use of loaded red blood cells, Stamler et al. merely states that "alternatively, nitrosothiols can be used to treat P. falciparum within red blood cells" without any example or supporting data. The Examiner further alleges that the treatment disclosed in Stamler et al. is to reduce or ameliorate abnormal parasitized red blood cell adhesion *in vivo* (column 5, lines 32 to 40). However, the cited patent states only "abnormal red blood cell adhesion" at column 5, line 32 to 40 and illustrates the point later by reference to sickle cell anaemia. Sickle cell anaemia is not the same as malaria. In the field of malaria, at the priority date although it was known that NO can kill *P. falciparum in vitro* it was thought that NO contributes to the pathology of cerebral malaria *in vivo*. It was not known whether or not NO protects infected subjects from the symptoms of severe malaria, nor does the citation provide any evidence that it does.

Based on the foregoing, Applicants submit that the invention as claimed is not anticipated by neither Rockett et al. nor Stamler et al. Withdrawal of the rejection under 35 U.S.C. 102 is respectfully requested.

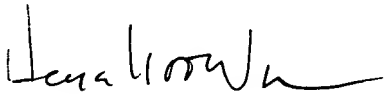
Conclusion:

In view of the foregoing, it is submitted that this case is in condition for allowance, and passage to issuance is respectfully requested.

If there are further issues related to patentability, the courtesy of a telephone interview is requested, and the Examiner is invited to call to arrange a mutually convenient time.

This amendment is accompanied by a Petition for Extension of Time (3 months) and a check in the amount of $510 as required under 37 C.F.R. 1.17. It is believed that this amendment does not necessitate the payment of any additional fees under 37 C.F.R. 1.16-1.17. If the amount submitted is incorrect, however, please deduct from Deposit Account No. 07-1969 the appropriate fee for this submission and any extension of time required.

Respectfully submitted,



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Molecular mechanisms of cytoadherence in malaria

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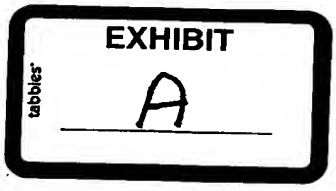

American Journal of Physiology - Cell Physiology

invited review

Molecular mechanisms of cytoadherence in malaria

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Ho, May, and Nicholas J. White. Molecular mechanisms of cytoadherence in malaria. *Am. J. Physiol.* 276 (*Cell Physiol.* 45): C1231–C1242, 1999.—Microbial pathogens subvert host adhesion molecules to disseminate or to enter host cells to promote their own survival. One such subversion is the cytoadherence of *Plasmodium falciparum*-infected erythrocytes (IRBC) to vascular endothelium, which protects the parasite from being removed by the spleen. The process results in microcirculatory obstruction and subsequent hypoxia, metabolic disturbances, and multiorgan failure, which are detrimental to the host. Understanding the molecular events involved in these adhesive interactions is therefore critical both in terms of pathogenesis and implications for therapeutic intervention. Under physiological flow conditions, cytoadherence occurs in a stepwise fashion through parasite ligands expressed on the surface of IRBC and the endothelial receptors CD36, intercellular adhesion molecule-1 (ICAM-1), P-selectin, and vascular adhesion molecule-1. Moreover, rolling on ICAM-1 and P-selectin increases subsequent adhesion to CD36, indicating that receptors can act synergistically. Cytoadherence may activate intracellular signaling pathways in both endothelial cells and IRBC, leading to gene expression of mediators such as cytokines, which could modify the outcome of the infection.

Plasmodium falciparum; erythrocyte; adhesion molecules; pathogenesis

ADHESION MOLECULES of the immune system are surface receptors that facilitate cell-cell interaction. They are central to the recruitment and trafficking of immune cells and thus in the generation of an immune response. Many of the adhesive molecules are constitutively expressed, whereas others may be induced or upregulated by cytokines and microbial products. In the past decade, it has become increasingly recognized that some microbial pathogens subvert these molecules to promote their dissemination or entry into host cells and thus defend against host immune responses. Examples of such subversion have been found in three major classes of microbial pathogens, i.e., viruses, bacteria, and parasites, and involve the integrins, selectins, and members of the immunoglobulin superfamily. Understanding the molecular events involved in these adhesive interactions is important in terms of both pathophysiology and implications for therapeutic intervention. In this paper, the molecular basis of the adhesive interactions between *Plasmodium falciparum*-infected erythrocytes (IRBC) and vascular endothelium is reviewed in the context of the clinical disease.

MALARIA

Malaria is the most important parasitic infection, affecting an estimated 350–500 million people worldwide and resulting in between 0.5 and 2 million deaths annually (110). Human malaria is caused by one of four *Plasmodium* species, namely, *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*. The life cycle of malaria parasites is complex, with asexual reproduction occurring in the mammalian host and sexual reproduction in the anopheline mosquito vectors (Fig. 1) (107). The parasites are transmitted to humans in the form of sporozoites through the bite of a female anopheline mosquito. The sporozoites circulate for up to 45 min before entering hepatocytes, in which they undergo asexual reproduction to form a large intracellular schizont. Hepatic schizonts contain thousands of merozoites when they are mature (within 5–15 days of sporozoite inoculation). The swollen hepatocyte eventually bursts, discharging merozoites into the bloodstream, where they rapidly invade erythrocytes to initiate the erythrocytic cycle. In *P. vivax* and *P. ovale* infections, dormant liver forms also occur that are capable of reactivating

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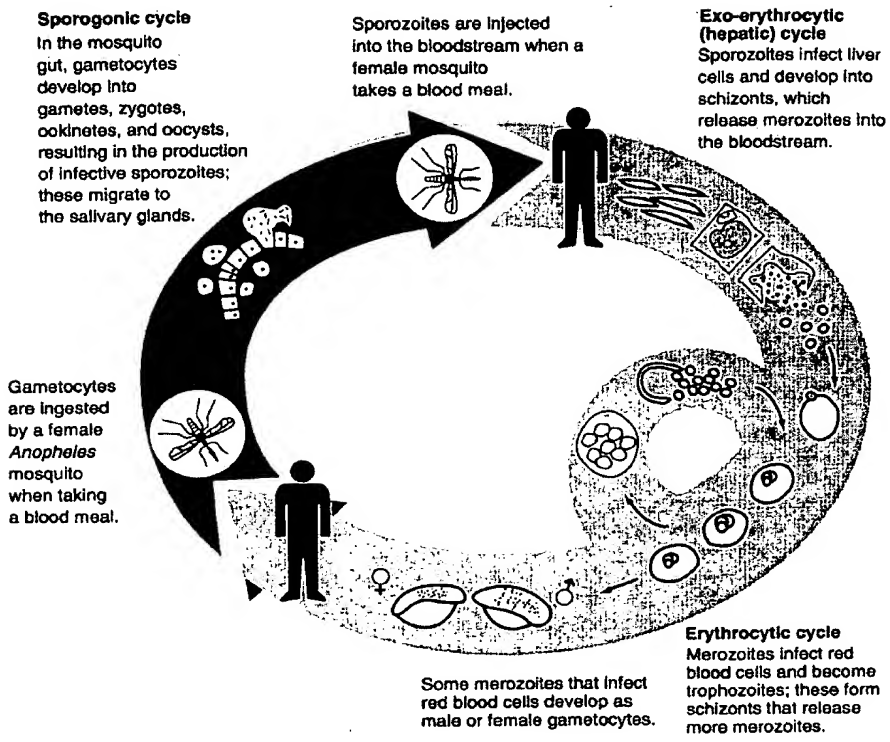

Fig. 1. The malaria transmission life cycle. [From White and Breman (107), with permission of The McGraw-Hill Companies.]

and forming a hepatic schizont weeks or months later. Merozoite attachment to the red blood cell is mediated via a specific erythrocyte surface receptor. Inside the erythrocyte, the parasite develops within a membrane-bound parasitophorous vacuole first as a trophozoite and then, during multiple nuclear division known as schizogony, as a schizont. When the schizont matures, the host red blood cell ruptures, liberating merozoites that rapidly invade fresh erythrocytes in the general circulation, thus continuing the life cycle. Some merozoites develop into sexual forms (gametocytes), which are taken into the mosquito gut with a blood meal. These may fuse forming a zygote and then undergo meiosis to form first an ookinete and later an oocyst in the gut wall. This phase of multiplication in the mosquito is known as sporogony. The oocyst also bursts, liberating large numbers of sporozoites that migrate to the salivary glands to await injection into the human host during the next blood meal.

P. falciparum is the causative agent of the most severe of the four infections and accounts for most of the mortality. Most deaths occur in children under five years of age (110). Falciparum malaria is an acute febrile illness characterized by fever, chills, headache, anemia, and splenomegaly, which responds promptly to appropriate antimalarial therapy. Untreated, the patient will either die in the acute attack (<5%) or survive with almost no development of immunity against the next infection unless he or she has been exposed to years of repeated challenge. Approximately 5–10 million infected individuals per year develop complications during the acute infection, manifested as coma (cerebral malaria), metabolic acidosis, hypoglycemia, severe anemia, and, in adults, renal failure and pulmonary edema (108). The overall mortality from severe malaria varies from 15 to 30%, with the highest mortality resulting from cerebral malaria, metabolic acidosis, and pulmonary edema.

CYTOADHERENCE

The pathogenicity of *P. falciparum* results from its potential to multiply to high parasite burdens and the unique ability to adhere to capillary and postcapillary venular endothelium during the second half of the 48-h life cycle, a process that is called cytoadherence (52, 53). The resulting sequestration of infected erythrocytes (IRBC) leads to alterations in microcirculatory blood flow, metabolic dysfunction, and, as a consequence, many of the manifestations of severe falciparum malaria (Fig. 2). Cytoadherence confers at least two survival advantages for the parasites: the microaerophilic venous environment is better suited for their maturation, and adhesion to endothelium allows them to escape clearance by the spleen, which recognizes their loss of deformability (26, 49) and opsonization with antibodies and/or complement components (40).

As a result of cytoadherence, patients who die in the acute phase of falciparum malaria have intense sequestration of erythrocytes containing mature forms of the parasite in the microvasculature of vital organs (1, 53, 68). The organ distribution of sequestration varies and

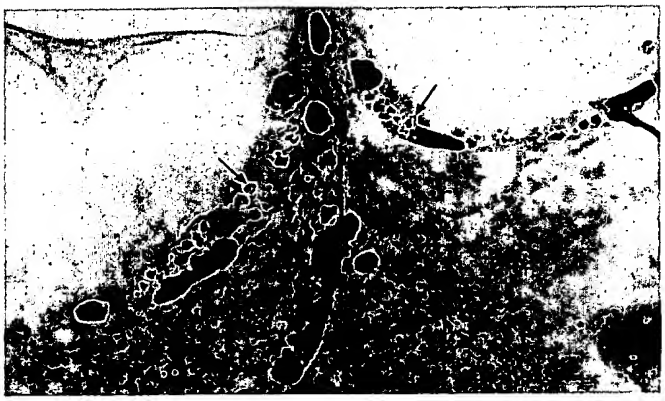

Fig. 2. Cerebral venules packed with infected erythrocytes (arrows) in a fatal case of cerebral malaria.

tends to reflect the clinical features of the preceding clinical illness; for example, patients with coma (cerebral malaria) show increased cerebral sequestration compared with that in other organs (76, 81). Even within the brain, variation in the degree of sequestration is seen between cerebral and cerebellar vessels, and white and gray matter (89). At the microvascular level, there is considerable heterogeneity among the individual vessels (N. J. White and K. Silamut, unpublished observations). Some are packed with IRBC containing fully developed schizonts, others with mature trophozoites that have yet to undergo schizogony, and others contain no parasites. This synchronous clustering suggests that, once the erythrocytes have adhered, "unsticking" and recirculation do not occur. The majority of parasites in fatal cases are at the mature trophozoite stage, but this probably represents antemortem drug effects that preferentially arrest development at this stage. In the acute phase of cerebral malaria there is remarkably little extravascular pathology, and, although occasional fibrin strands may be seen, platelets are also notable by their absence (53). Inflammatory cells are more prominent in patients who die many days after starting treatment and in whom parasites have largely cleared, and phagocytic cells are seen to be ingesting parasite pigment that has been released by ruptured IRBC or that remains in cytoadherent erythrocyte ghosts. There is no pathological evidence of acute vasculitis.

At the ultrastructural level, electron-dense, knoblike protrusions of the erythrocytic membrane are seen at the points of contact between the IRBC and endothelial cells (Fig. 3) (42, 53, 68). These knobs are made up of parasite-encoded proteins that have been exported to the surface of the infected erythrocyte. They are essential for firm cytoadherence by facilitating the initial attachment of the infected erythrocyte to the endothelial cell and by concentrating the parasite ligands at a particular site. Although knobless IRBC can adhere to target cells in static binding assays in vitro (16, 102), ultrastructural studies of human tissues from fatal malaria cases have not shown cytoadherence independent of knobs. Furthermore, targeted disruption of the gene encoding one of the knob proteins, knob-associated histidine-rich protein (KAHRP or PfHRP1), results in the failure of knob formation and an inability of the IRBC to adhere under flow conditions in vitro (24). Thus these knobs appear to serve the same function as microvilli on the surface of leukocytes, where ligands for interaction with endothelium are presented (58). Parasitism would continuously favor the selection of knob-positive organisms able to form a more stable union with host cells and thus to allow the parasites to evade splenic clearance, thereby increasing the probability of survival and transmission.

ENDOTHELIAL RECEPTORS

The stage and host cell specificity of cytoadherence suggests that the process involves specific parasite or host ligands expressed on the surface of IRBC and vascular endothelium. A number of endothelial receptor molecules have been identified, based on their ability to support the adhesion of laboratory-selected parasite lines and clones in static adhesion assays in vitro. The first of these molecules described was thrombospondin (TSP) (82). IRBC adhere to immobilized TSP in a dose-dependent manner but not to other adhesive proteins such as fibronectin or von Willebrand factor. Subsequent investigations showed that, although TSP may contribute to cytoadherence, it is not sufficient to mediate the process by itself. IRBC do not adhere to every cell line that secretes TSP (72), and anti-TSP antibodies do not inhibit cytoadherence to C32 melanoma cells that express CD36 and intercellular adhesion molecule-1 (ICAM-1) in addition to TSP (66).

The second receptor molecule to be implicated in cytoadherence was CD36 (7, 8, 66). CD36, or platelet glycoprotein IV, is found on monocytes, endothelial cells, platelets, and erythroblasts. Its natural ligands include collagen (98), TSP (5), and both the natural and oxidized forms of high-density lipoproteins, low-density lipoproteins, and very-low-density lipoproteins (21). A

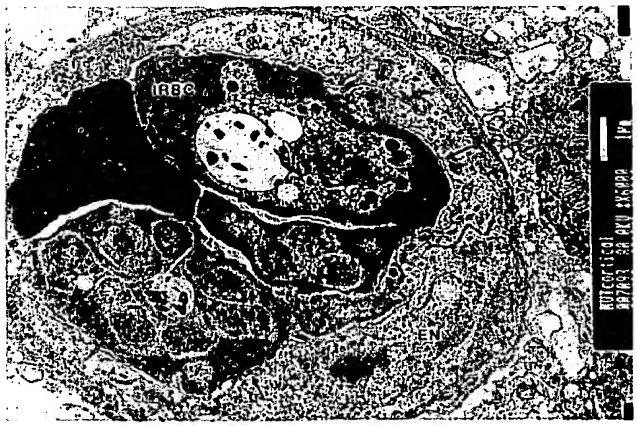

Fig. 3. Cytoadherence between infected erythrocytes (IRBC) and endothelial cells (EN) showing knobs (arrows) at the points of attachment. [From E. Pongponratn and D. Ferguson, with permission (unpublished observations).]

monoclonal antibody to CD36, OKM5, inhibits and reverses the cytoadherence of IRBC to a number of target cells in vitro, including dermal microvascular endothelial cells and C32 melanoma cells, as well as purified CD36 immobilized on plastic. Furthermore, although IRBC binding to TSP and CD36 is highly correlated, IRBC can adhere directly to CD36-transfected COS cells in the absence of TSP (69). With the use of peptides generated from a random CD36 domain library, it has been shown that IRBC interact with sites on the CD36 molecule that are distinct from the binding sites of TSP (6). The fact that OKM5 blocks the adhesion of all parasite isolates tested so far suggests that all IRBC bind to a common region on the molecule. The interaction of IRBC with CD36 expressed on monocytes leads to a respiratory burst (64), with the production of oxidative metabolites that are toxic to intraerythrocytic parasites (65).

A third endothelial molecule that acts as a receptor for IRBC selected on human umbilical vein endothelium (HUVEC) is ICAM-1 (13), which belongs to the immunoglobulin superfamily of adhesion molecules. ICAM-1 is a glycoprotein that acts as a ligand for the leukocyte integrin lymphocyte function-associated antigen-1 (LFA-1) and plays a central role in the generation of an immune response (86). It is distributed widely on venular endothelium, where it has been shown to be crucial for neutrophil adhesion before the transmigration of these cells into an inflammatory focus (91). ICAM-1 expression on endothelial cells can be upregulated by the proinflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and interferon-γ (IFN-γ) (75). ICAM-1 is also the receptor for human rhinoviruses (92). Molecular studies have shown that IRBC binds to the first immunoglobulin domain of ICAM-1 at a site that is 180° from the binding sites of both LFA-1 and rhinovirus (12, 61).

Two other endothelial receptors have been shown to mediate the cytoadherence of a parasite clone that was derived from a clinical parasite isolate by sequential panning of IRBC on purified CD36, ICAM-1, E-selectin, and vascular adhesion molecule-1 (VCAM-1) proteins (67). VCAM-1 is not expressed constitutively on endothelial cells but can be induced by TNF-α, IL-1, and IL-4 on endothelium with kinetics similar to ICAM-1 (70, 71). Its interaction with its ligand, the β_1-integrin very late antigen-4 (VLA-4; $\alpha_4\beta_1$), provides a second lymphocyte-endothelium adhesion mechanism distinct from that of LFA-1 and ICAM-1. There is increasing evidence that VCAM-1 can mediate all three phases of leukocyte-endothelial cell interaction i.e., tethering, rolling, and adhesion (3, 14, 44). Neutrophils (80) and sickle reticulocytes (95) also adhere to VCAM-1 via VLA-4.

IRBC from the above parasite clone also adhere to E-selectin. The selectins are a family of differentially expressed adhesive molecules that recognize fucosylated and sialylated carbohydrate ligands (45). Both P-selectin and E-selectin are intimately involved in the initial interaction of neutrophils at the onset of acute inflammation. E-selectin is not constitutively expressed on endothelial cells but is induced (peak 4–6 h) after exposure to IL-1 and TNF-α (75). It supports the rolling of neutrophils on vascular endothelium in vitro (47) but probably at a later stage of inflammation than P-selectin, which can be quickly mobilized from an intracellular store in Weibel-Palade bodies (36).

More recently, chondroitin-4-sulfate (CSA), a glycosaminoglycan expressed throughout the microvasculature in association with various proteoglycans such as thrombomodulin, has been shown to mediate the cytoadherence of IRBC selected on Chinese hamster ovary cells (85). CSA is now recognized as the principal molecule mediating cytoadherence in the human placenta, where it is expressed on syncytiotrophoblasts (31). The unique interaction between IRBC and CSA in the placenta is discussed in CYTOADHERENCE IN THE PLACENTA.

STUDIES OF CLINICAL PARASITE ISOLATES

There are some inherent problems in extrapolating from results obtained in vitro with selected parasite lines and clones directly to malaria parasites causing clinical infections. The cytoadherent phenotype is known to be influenced by long-term culture in vitro (28, 100) and by the selection and cloning process itself (15). To determine the relative importance of the above receptor molecules for wild-type parasites, the cytoadherence of IRBC taken directly from the peripheral blood of patients with acute falciparum malaria has been examined in a number of studies using static binding assays (22, 35, 39, 60, 62, 105). These heterogeneous populations of IRBC are cultured in vitro for 24–36 h (i.e., less than one asexual cycle) until they reach the cytoadherent trophozoite/schizont stages. Greater than 90% of clinical parasite isolates tested adhere to CD36, whereas ~10% adhere to ICAM-1. For isolates that adhere to both molecules, the degree of adhesion to CD36 is at least 10-fold higher than adhesion to ICAM-1. Minimal or no adhesion to E-selectin, VCAM-1, or CSA is seen with most of the isolates. The cytoadherence to TSP, C32 melanoma cells, and purified CD36 is found to be directly proportional to parasitemia (35, 39), expressed as the percent of infected erythrocytes. When the degree of cytoadherence to CD36 is compared at a fixed parasitemia, a range of intrinsic cytoadherent capabilities among different isolates becomes evident and in some instances correlates positively with the clinical severity of the infection (39, 62).

Immunopathological studies of postmortem tissues have also been performed to assess IRBC-endothelium interactions. In at least one study, colocalization of sequestration with ICAM-1 expression was noted, particularly in the brain (99). This finding may be interpreted as suggesting that ICAM-1 is the principal receptor for cytoadherence in the cerebral circulation. However, postmortem studies need to be interpreted with caution, as adhesion molecules could be expressed as a consequence of the circulatory disturbance and metabolic abnormalities resulting from cytoadherence rather than being its cause. For example, oxidant damage by adherent sickle cells has been shown to induce the expression of ICAM-1, E-selectin, and VCAM-1 on vascular endothelium (94).

From a population perspective, results from static binding assays suggest that ~30% of IRBC in a given parasite isolate adhere to CD36, whereas 2–3% of IRBC are adherent to ICAM-1 (105). The percentage of IRBC adherent to E-selectin and VCAM-1 is negligible. This means that >60% of IRBC do not bind to any of the receptor molecules in vitro. There are several possible explanations for the in vitro observations. On the parasite side, not all IRBC may express the parasite proteins that are involved in cytoadherence. So-called "null" cells can appear after long-term culture in vitro (28), but their presence in overnight culture of wild-type parasites has not been shown. A high percentage of null cells is also unlikely in vivo, in that mature parasites are seldom seen in the peripheral blood, yet multiplication is remarkably efficient even in the face of splenic clearance. There may be other receptors for cytoadherence that have not been identified. Recent studies have suggested that platelet/endothelial cell adhesion molecule-1 may also have a role in mediating cytoadherence of clinical isolates (60). However, the minor degree of adhesion to this molecule would not account for the cytoadherence of the majority of the IRBC in a given isolate. A more likely scenario is that IRBC may need to interact with a number of endothelial receptor molecules for optimal adhesion. The interaction with some of the adhesion molecules such as E-selectin and VCAM-1, and possibly P-selectin, may be revealed only under flow conditions. To ensure its own survival, IRBC will likely use any readily available adhesion molecule to adhere to the vascular endothelium in vivo.

CYTOADHERENCE UNDER FLOW CONDITIONS

To address the above possibilities, studies have been performed using a parallel plate flow microscopy system that allows the interaction between IRBC and receptor molecules to be visualized directly under physiological flow conditions (104). IRBC from clinical parasite isolates were observed to interact with endothelial receptors in a stepwise process that involves tethering, rolling, and firm adhesion (Fig. 4). IRBC initially tether and then roll on CD36, ICAM-1, P-selectin, and VCAM-1. However, the strength of the rolling interaction with each receptor molecule varies, as reflected in differences in rolling velocity, and significant adhesion under shear is almost exclusively to CD36. Some IRBC bypass the rolling event and are arrested on CD36 immediately after tethering. There is no interaction with E-selectin under flow conditions.

The interaction between IRBC and P-selectin has been further characterized using this methodology (37). P-selectin is stored in Weibel-Palade bodies of endothelial cells and is rapidly translocated to the cell surface in response to various mediators including oxidants, histamine, thrombin, and cysteinyl leukotrienes (36). P-selectin may be important in mediating the capture and fast rolling of IRBC followed by slower rolling and attachment to CD36. The IRBC-P-selectin interaction is Ca^{2+} dependent and involves a sialic acid residue on IRBC. However, the monoclonal antibody G1, which inhibits the interaction between neutrophil and P-selectin, has no effect on IRBC rolling on P-selectin. This finding indicates that the primary binding site for IRBC on P-selectin is different from the epitope responsible for the binding of its natural counterreceptor P-selectin glycoprotein ligand-1 (58).

On C32 melanoma cells, which coexpress CD36 and ICAM-1, inhibition of rolling by an anti-ICAM-1 antibody reduces the subsequent adhesion of some parasite isolates to CD36. Similarly, inhibition of rolling by an anti-P-selectin antibody reduces IRBC adhesion to CD36 on activated platelets. The rolling interactions with molecules such as ICAM-1 and P-selectin appear to facilitate adhesion to CD36, even if they individually are of much lower avidity than that required to allow attachment. This is the first demonstration of synergism among receptor molecules for cytoadherence under flow conditions. There is also synergism between CD36 and ICAM-1 in mediating cytoadherence to human dermal microvascular endothelial cells under static conditions (56). Collectively, these results indicate that cytoadherence under physiological flow conditions may be mediated by multiple IRBC ligands that interact with different adhesion molecules in a cooperative fashion. The synergism may be particularly important in view of the fact that CD36 expression on microvascular endothelium does not appear to be upregulatable (74). In other words, the degree of cytoadherence of *P. falciparum* on vascular endothelium may be regulated at the level of expression of adhesion molecules such as P-selectin and ICAM-1 rather than that of CD36.

On the other hand, there are also parasite isolates that appear to be able to interact exclusively with CD36 on either C32 cells or platelets. These results highlight

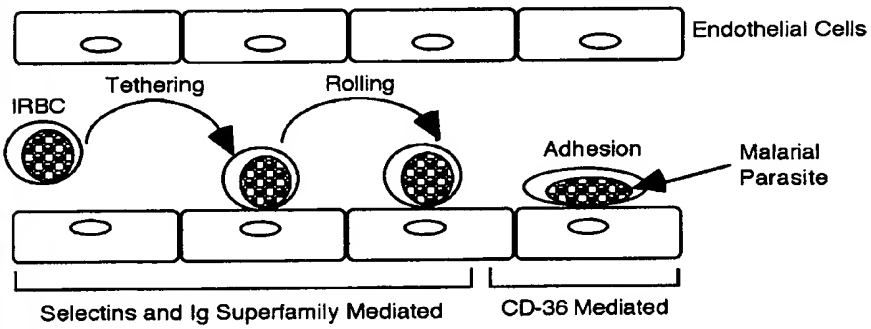

Fig. 4. Schematic diagram of the different phases of IRBC interaction with endothelium observed under physiological flow conditions.

the diversity of the adhesive properties of clinical *P. falciparum* isolates relative to the more homogeneous adhesive mechanisms used by leukocytes. There are other striking differences between the adhesive interactions of IRBC and leukocytes with endothelial receptors. First, adhesion of IRBC to CD36 can occur without prior interaction with another molecule, whereas leukocytes do not adhere directly. Second, ICAM-1 acts as a receptor for firm adhesion of neutrophils but cannot tether or support rolling of these cells except at extremely low shear. In contrast, ICAM-1 mediates the tethering and rolling of IRBC. Third, leukocytes require activation after the initial interaction with endothelial receptors, which permits subsequent firm adhesion. However, IRBC are able to roll and adhere firmly to endothelial receptors without notable activation or the obvious requirement for chemotactic molecules, because the adhesive interactions can occur on formalin-fixed transfectants expressing the adhesion molecules (105) or endothelial cells (23).

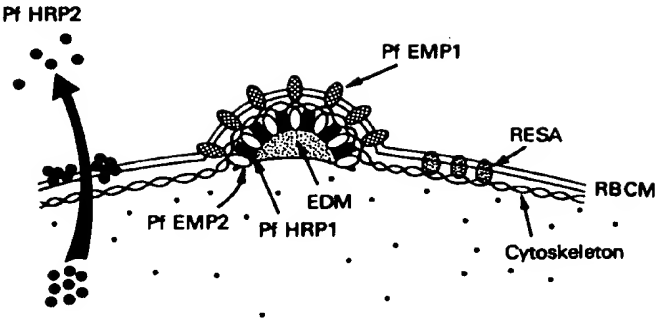

Fig. 5. Schematic diagram of topological distribution of *P. falciparum* proteins in surface membrane of infected erythrocytes. Lipid bilayer of red blood cell membrane (RBCM) is indicated, together with the cytoskeleton and electron-dense material (EDM) under knobs. PfEMP, *P. falciparum* erythrocyte membrane protein; PfHRP, *P. falciparum* histidine-rich protein [also known as knob-associated histidine-rich protein (KAHRP)]; RESA, ring-infected erythrocyte surface antigen. [From Howard (42), reproduced with permission of S. Karger AG, Basel.]

CYTOADHERENCE IN THE PLACENTA

Falciparum malaria adversely affects pregnancy (17, 18). In areas of intense transmission, where symptomatic disease in adults is rare, these adverse effects are largely confined to the fetus of the first pregnancy (57). There is intrauterine growth retardation and a reduction in birth weight (18). Infections with *P. falciparum* in nonimmune pregnant women tend to be severe (50). Pregnant women with cerebral malaria have a case mortality rate over twice that of nonpregnant adults. Premature labor and fetal death are common.

The placenta is a site of preferential IRBC accumulation (19), but on microscopic examination this sequestration appears to occur in the absence of cytoadherence, because IRBC are seen free in the intervillous space and not adherent to endothelial surfaces (54). Experimental evidence has shown that, although CD36 appears to be the main receptor for IRBC on venular endothelium, parasites obtained directly from human placentas adhere to CSA expressed on syncytiotrophoblasts that line the placental intervillous space (31). The CSA may be presented on proteoglycans that extend well beyond the cell surface. Moreover, immune sera from multigravid women but not males or primigravid women inhibit cytoadherence to CSA (32). The presence of anti-CSA antibodies may well explain why the falciparum-malaria-associated reduction in birth weight is lower or absent in women with multiple pregnancies.

PARASITE CYTOADHERENT LIGANDS

At least five parasite-derived proteins are associated with the cell membrane of an infected erythrocyte at various stages of the developmental cycle (Fig. 5) (42). Three of the malarial proteins are associated with knobs [*P. falciparum* erythrocyte membrane proteins 1 and 2 (PfEMP1 and PfEMP2) and PfHRP1 or KAHRP]. Of these, PfEMP1 is the only protein that extends beyond the cell surface to mediate cytoadherence (55), whereas PfEMP2 and PfHRP1 or KAHRP remain on the internal face of the erythrocyte membrane in association with electron-dense material. The proteins appear to be exported from the intracellular parasite through the erythrocyte cytoplasm to the surface membrane via a complex system of vesicle trafficking pathways (77).

PfEMP1 consists of a family of diverse-size (200–350 kDa) surface proteins that are linked to the erythrocyte cytoskeleton. The protein is encoded by a large family of *var* genes. There are ~50–150 copies of the *var* gene per genome and a different set of *var* genes in each *P. falciparum* clone (92). Several members of the family of *var* genes that encode PfEMP1 have been cloned. The protein has four Duffy-binding-like (DBL) domains, each containing five consensus motifs rich in cysteine residues (11, 93). Three cysteine-rich motifs, denoted CRM-1 to CRM-3, are located after DBL-1, DBL-2, and DBL-4, respectively. The presence of an RGD motif (amino acids 1212–1214) and an LDV motif (amino acids 142–144) in the extracellular domain suggests that PfEMP1 may participate in ligand-receptor interactions involving the integrin family. There is a single transmembrane domain, followed by a presumed intracellular domain encoded by the 3′ exon. Tryptic fragments of PfEMP1 cleaved from the surface of IRBC bind to CD36, ICAM-1, and TSP (9). The epitope on PfEMP1 that interacts with CD36 has been mapped to the rC1–2 region of CRM-1, which corresponds to amino acids 576–808 (10). Recently, CSA-adherent parasites have been shown to transcribe a particular *var* gene with the production of a distinct variant PfEMP1, and inhibition studies with antibodies raised against different domains of the protein suggest that the binding site may be located on DBL-3 (79).

In addition to variations in molecular size, PfEMP1 undergoes a high rate (~2% per cycle) of antigenic variation in vitro (83), with resultant changes in the cytoadherent phenotype. This considerable antigenic variation could contribute to immune evasion and thus

to the survival of the blood stage infection. Whether this degree of antigenic plasticity occurs in vivo has yet to be firmly established. Studies of clinical parasite isolates would suggest that CD36 exerts a strong selective pressure on the IRBC cytoadherent phenotype of these heterogeneous parasite populations. Recent data in isogenic *P. falciparum* populations with defined adhesive phenotypes for CD36, ICAM-1, and CSA by selective panning indicate that multiple *var* genes may be transcribed early in the parasite cycle. However, a silencing mechanism appears to come into play as the parasite matures, so that only a specific *var* gene is transcribed in trophozoites (85).

A molecule similar to PfEMP1, of molecular mass 270 kDa, called sequestrin, has been demonstrated on the surface of infected erythrocytes using anti-idiotypic antibodies raised against OKM8, a monoclonal antibody for CD36 (63). This finding further strengthens the hypothesis that CD36 is the major receptor on vascular endothelium for the parasite cytoadherent ligand.

Two other parasite proteins, ring-infected erythrocyte surface antigen (RESA) and PfHRP2, are associated with the erythrocyte membrane but are not localized specifically to the knobs (Fig. 5). PfHRP2 is secreted into the circulation and is currently being exploited for diagnostic assays. The 155-kDa RESA antigen is transferred from the merozoite to the erythrocyte membrane during invasion (2). Once transferred, it is thought to be entirely submembranous. RESA has been shown to have cross-reactive epitopes with band 3 protein (41), the human erythrocyte anion transporter, and this too has been implicated as a ligand for cytoadherence mediated by CD36 (26). Presumably, changes in the erythrocyte cytoskeleton that occur as a result of parasitization could expose previously cryptic host molecules (neoantigens) on the cell surface.

A few other cytoadherent ligands remain to be defined. The sialylated P-selectin ligand is trypsin sensitive, as is PfEMP1. The major ligand for VCAM-1 is VLA-4 ($\alpha_4\beta_1$). VLA-4 has been shown to mediate the adhesion of sickle reticulocytes to endothelial cells (95). IRBC may make use of the same mechanism. VLA-4 is expressed by late erythroid progenitors and erythroblasts but is not detectable on mature erythrocytes (73). It is conceivable that VLA-4 may be reexposed on the surface of the erythrocyte due to topographical changes induced by the maturing intracellular parasite or that IRBC may express a VLA-4-like ligand that is of parasite origin. An LFA-1-like ligand has been demonstrated on one clinical *P. falciparum* isolate, and an anti-LFA-1 monoclonal antibody partially inhibited its adhesion to cytokine-stimulated cerebral microvascular endothelium (96).

Regardless of the eventual identity of the cytoadherent ligand(s), a conserved component must be present, since all *P. falciparum* parasite isolates causing natural infections cytoadhere. In addition, there must be a strain-variable component, since inhibition or reversal of cytoadherence by immune sera occurs in a strain-specific manner (101). The constant and variant components could be either closely associated molecules or different epitopes on the same molecule.

CYTOADHERENCE AND SIGNALING

The functional consequences of the adhesion process have been little studied. It is now well recognized that adhesion molecules are not just innocent bystander molecules that function only to mediate leukocyte interactions with endothelium but that they also initiate signal(s) that can activate and regulate adhesion-dependent leukocyte function, β_2-integrin activation, O_2^- production, and T cell receptor stimulation (27, 51). A functional consequence of cytoadherence might be the activation of intracellular signaling pathways in the endothelial cell, leading to changes in the status of the adhesion molecules and/or gene expression of immunoregulatory molecules such as cytokines and nitric oxide synthase, which would modify the outcome of the infection. The triggering of downstream events by cytoadherence would be consistent with the observation of a respiratory burst following adhesion of IRBC to CD36 on monocytes (64). In this connection, it is interesting to note that CD36 is present in a subcompartment in the plasma membrane known as caveolae, which are present on endothelial cells. Caveolae are flask-shaped invaginations that have been proposed to represent a site of signal transduction. Although the exact mechanism by which the signal is activated is unknown, the *src* family kinases have been shown to colocalize in caveolae (48) and have been shown to coprecipitate with CD36 (29).

Signal transduction might also be induced in erythrocytes, even though they are often considered biologically inactive cells. Signal-dependent translation of a number of proteins has been described in activated human platelets, which, like erythrocytes, lack nuclei, cannot synthesize mRNA, and are considered incapable of regulating protein synthesis (106). More importantly, signal transduction might involve the metabolically active parasite via a putative parasitophorous duct pathway that allows for the transport of macromolecules directly from the environment into the parasite (78). A glycosylphosphatidylinositol (GPI) anchor moiety of *P. falciparum* has been shown to induce nitric oxide synthase expression in HUVEC by a protein tyrosine kinase-dependent and protein kinase C-dependent signaling pathway (97). The toxin also upregulates ICAM-1, VCAM-1, and E-selectin expression in HUVEC via tyrosine kinase-dependent signal transduction (88).

MODULATION OF CYTOADHERENCE

The ultimate goal of any research on cytoadherence is the development of safe and effective methods of inhibiting or reversing the process. Soluble mediators such as cytokines, chemokines, nitric oxide, and certain drugs are known to regulate adhesion molecule expression and thus may modulate cytoadherence. Furthermore, these agents may have direct effects on IRBC or endothelial cells.

The effect of cytokines on cytoadherence is well documented. TNF-α, IL-1, and IFN-γ enhance the adhesion of IRBC from laboratory-adapted parasites to endothelial cells under static conditions (13, 43). These cytokines are known to be elevated in patients with *P. falciparum* malaria (34, 46). However, the effect of the cytokines on the rolling and adhesion of clinical parasite isolates to microvascular endothelium under shear stress has not been determined. Furthermore, the effect of other cytokines, either singly or in combination with the proinflammatory cytokines, is unknown. The effect of IL-10, an immunosuppressive cytokine produced by Th2 cells as well as monocytes and B cells, is of particular interest, because it inhibits TNF-α, IL-1, and IL-6 production by peripheral blood mononuclear cells in response to malarial antigens in vitro (38). It has also been shown to downregulate ICAM-1 expression induced by IFN-γ on monocytes (109). In fatal falciparum malaria, there appears to be a defect in the IL-10 control of proinflammatory cytokine production (N. P. Day and M. Ho, unpublished observations). IL-4 is also produced by some patients in response to *P. falciparum*, and it may contribute to cytoadherence by upregulating VCAM-1 expression (71).

Chemokines are small proinflammatory peptides that have a crucial role in the mobilization of the cells of the immune system through their leukocyte chemoattractant activity. The cytoadherence of IRBC to formalin-fixed target cells suggests that the process can occur in the absence of any chemotactic agents in vitro. However, this does not exclude a role for chemokines in enhancing cytoadherence in vivo. Human erythrocytes as well as postcapillary venular endothelial cells express the promiscuous Duffy antigen receptor (DARC) that binds to chemokines of both the C-X-C (e.g., IL-8, MGSA/gro, NAP-2) and C-C (e.g., RANTES, MCP-1) classes with high affinity (59). The presence of DARC on erythrocytes suggests that IRBC may respond to a chemotactic gradient. Plasma chemokine levels, including IL-8 and MIP-1, are elevated in falciparum malaria (20, 33).

Nitric oxide has been postulated to have both pathogenic and protective roles in severe falciparum malaria. Nitric oxide could derive from different synthetic enzymes in neuronal tissue or vascular endothelium or from an inducible form found in phagocytic cells. In acute malaria, increased nitric oxide production would be expected as a result of the proinflammatory response and microcirculatory ischemia. Moreover, nitric oxide synthase expression in endothelial cells can be induced by a GPI toxin from *P. falciparum* (97). Nitric oxide derivatives have been shown to inhibit the growth of *P. falciparum* in vitro (84). Nitric oxide may exert an inhibitory effect on IRBC rolling and adhesion, just as it inhibits neutrophil rolling on endothelial cells in models of ischemia reperfusion (30) and platelet aggregation (90). In patients with malaria, an inverse relationship between the severity of the infection and nitric oxide production/nitric oxide synthase expression has been demonstrated by some (4) but not others. The precise role of this evanescent molecule in host protection against malaria, vasoregulation, and the pathogenesis of cerebral malaria remains to be determined.

Last, antimalarials have been shown to inhibit cytoadherence at concentrations that inhibit overall nucleic acid and protein synthesis (103). Drugs that affect cytoadherence specifically have not been described. We have observed that, with suboptimal doses of the iron chelator desferrioxamine, the degree of inhibition of adhesion to CD36 consistently exceeds overall growth inhibition, suggesting that an additional mechanism may be involved in the activity of this drug against cytoadherence. Desferrioxamine has been shown to inhibit the transcription of several mitochondrial enzymes of *P. falciparum* (S. R. Meshnick, unpublished observations). The expression of the parasite cytoadherent ligand PfEMP1 may be similarly affected by environmental conditions such as low intraerythrocytic iron.

CONCLUSION

In human *P. falciparum* malaria infection, IRBC either sequester or are removed from the circulation primarily by the spleen. The balance between splenic clearance and sequestration, which allows the parasite to survive to initiate a new life cycle, is a major determinant of the rate of increase and magnitude of the infecting parasite burden. Within this paradigm, pathogenicity is proportional to the size of the sequestered parasite burden and the pattern of vital organ sequestration. In the past decade, detailed molecular studies have provided exciting new insight into the process of cytoadherence. The next challenge lies in translating the advances in our understanding of pathogenesis into improved treatment for the many millions who are affected by falciparum malaria.

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Chronic dietary supplementation with L-arginine inhibits platelet aggregation and thromboxane A_2 synthesis in hypercholesterolaemic rabbits in vivo

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Abstract

Objectives: L-arginine exerts anti-atherosclerotic effects in hypercholesterolaemic rabbits via modulating endogenous NO production. We investigated whether L-arginine inhibits thromboxane formation in vivo and platelet aggregation ex vivo in this animal model. **Methods:** The urinary excretion rates of 2,3-dinor-6-keto-$PGF_{1\alpha}$ (major urinary metabolite of PGI_2) and 2,3-dinor-TXB_2 (major urinary metabolite of thromboxane A_2) were used as indicators of platelet–endothelial cell interactions in vivo. Rabbits were fed 1% cholesterol (Cholesterol group, $N = 8$), 1% cholesterol plus 2,25% L-arginine (Cholesterol + L-arginine, $N = 8$), or normal rabbit chow (Control, $N = 4$) for 12 weeks. Urine samples were collected in weekly intervals. At the end of the study period platelet aggregation ex vivo and endothelium-dependent and -independent vascular function of isolated aortic rings in vitro was assessed. **Results:** Urinary 2,3-dinor-TXB_2 excretion significantly increased in the cholesterol group ($p < 0.05$), and endogenous NO formation (measured as urinary nitrate excretion) decreased ($p < 0.05$). Both parameters were significantly correlated with each other ($R = 0.48$, $p < 0.01$). L-arginine partly restored urinary nitrate excretion and significantly reduced TXA_2 production to values even below those in the control group ($p < 0.001$). Urinary 2,3-dinor-6-keto-$PGF_{1\alpha}$ excretion increased in early hypercholesterolaemia and returned to control values in the second half of the study period. The early increase in urinary 2,3-dinor-6-keto-$PGF_{1\alpha}$ excretion was attenuated by L-arginine. Platelet aggregation was significantly enhanced in cholesterol-fed rabbits and attenuated by dietary L-arginine. L-arginine also improved the impaired endothelium-dependent relaxations to ADP, and normalized the vasoconstrictor effects of 5-HT in isolated aortic rings. **Conclusions:** Cholesterol-feeding enhances platelet aggregation and TXA_2 formation, and stimulates platelet–endothelial cell interaction in rabbits. These effects are probably due to impaired NO elaboration, as indicated by decreased urinary nitrate excretion. Chronic dietary supplementation with L-arginine elevates systemic NO elaboration and significantly increases the PGI_2/TXA_2 ratio. It thus beneficially influences the homeostasis between vasodilator and vasoconstrictor prostanoids in vivo. © 1998 Elsevier Science B.V.

Keywords: Nitric oxide; Prostacyclin; Endothelium; Platelet aggregation; Gas chromatography–mass spectrometry

1. Introduction

Hypercholesterolaemia and atherosclerosis are characterized by impaired endothelium-dependent vascular relaxation [1] due to a reduced ability of the endothelium to elaborate biologically active nitric oxide (NO) [2] and, possibly, prostacyclin [3]. Besides their vasodilator effects, both NO and prostacyclin have been shown to inhibit platelet adhesion and aggregation [4,5]. This effect may be of importance for the atherosclerotic process, as activation of platelets at the sites of injured endothelium contributes to the progression of atherosclerosis in vivo [6]. Indeed,

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there is increased platelet aggregability in animal models of hypercholesterolaemia and in human subjects with familial hypercholesterolaemia [7,8]. Activated platelets also modulate the tone and the structure of the vascular wall by secreting a variety of vasoconstrictor and pro-proliferative mediators (e.g., thromboxane [TX] A_2, platelet-derived growth factor [PDGF], and serotonin [5-hydroxytryptamine, 5-HT]) [9,10]. Moreover, endothelium-dependent vasodilator responses to platelet mediators which induce vasodilatation in healthy blood vessels, like adenosine diphosphate (ADP), are decreased or converted into vasoconstriction in atherosclerotic arteries [11].

Several studies have investigated the production of thromboxane A_2 and/or prostacyclin in atherosclerosis [3,12]. However, the time course of changes in the formation rates of vasoactive prostanoids during the induction of atherosclerosis is unknown. It may be of importance, as prostacyclin is synthesized in part from endoperoxide precursors which are released from platelets and taken up by endothelial cells [5,13]. By this mechanism, increased platelet activation may also influence prostacyclin formation rates [14,15]. The balance between prostacyclin and thromboxane has therefore been used as an index of platelet–endothelial cell interactions in vivo [16].

The method of choice to reliably assess whole body formation rates of prostanoids is the quantitation of their main enzymatically formed urinary metabolites, i.e., 2,3-dinor-6-keto-$PGF_{1\alpha}$ and 2,3-dinor-TXB_2, by gas chromatography–tandem mass spectrometry (GC/MS/MS) [17,18], as these metabolites have been shown to be mainly derived from the endothelium and the platelets, respectively [17,19], and may be repeatedly and non-invasively analyzed. Similarly, endogenous NO synthesis rates can be assessed by quantifying the urinary excretion rate of nitrate, the oxidative metabolite of NO [20–22].

Chronic dietary administration of L-arginine, the precursor of endogenous NO, has been shown to improve endothelial function and to slow the progression of atherosclerosis in cholesterol-fed rabbits [20,23], probably by restoring the biological activity of endothelial NO.

The present study was undertaken to investigate whether dietary L-arginine affects thromboxane A_2 and/or prostacyclin synthesis in cholesterol-fed rabbits in vivo, as assessed using the GC/MS/MS technique to quantify the major urinary metabolites of TXA_2 (2,3-dinor-TXB_2) and prostacyclin (2,3-dinor-6-keto-$PGF_{1\alpha}$). The relation of these changes to changes in systemic NO formation was assessed by quantifying urinary nitrate excretion. Repetitive urine sampling allowed us for the first time to gain further insight into the time course of these changes during the induction of hypercholesterolaemia. Moreover, we studied whether attenuation of increased platelet aggregability was involved in the effects of L-arginine, and whether the vascular responsiveness to serotonin and ADP, potent platelet-derived vasoactive mediators, was modified by hypercholesterolaemia and by dietary L-arginine.

2. Material and methods

2.1. Study design

20 male New Zealand white rabbits were used for this study, which conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No 85-23, revised 1985) and had been approved by the local supervisory committee for studies in animals (Hannover). Two groups of 8 rabbits were fed rabbit chow enriched with 1% cholesterol (Altromin, Lage, Germany) and had free access to plain tap water (Cholesterol group) or water supplemented with 2.25% L-arginine (Sigma, Munich, Germany; Cholesterol + L-arginine group) for 12 weeks. This cholesterol feeding protocol has been shown previously to produce well-defined lesions throughout the thoracic and abdominal aorta with a marked thickening of the intima [24]. Another group of 4 rabbits was given normal rabbit chow and plain tap water (Control group). Before the beginning of the experimental period and during the feeding period, the rabbits were placed in metabolic cages at weekly intervals to collect 24-h urines. At baseline and the end of the dietary intervention, blood samples were drawn by puncture of the central ear artery into vacutainers containing 1.2 mg EDTA/ml whole blood for the measurement of plasma total cholesterol and L-arginine levels, and into vacutainers containing 3.13% sodium citrate (1:10, v/v) as anticoagulant for platelet aggregation ex vivo. Blood samples were obtained at the end of the urine sampling periods to avoid interference with urinary prostanoid excretion rates. The rabbits were sacrificed, and the aortas were excised for isometric tension recording.

2.2. Platelet aggregation

Platelet rich plasma (PRP) was generated from citrated blood by centrifugation at 200 g for 10 min. Platelet poor plasma (PPP) was prepared from the remaining volume of blood by centrifugation at 1600 g for 10 min. Platelet aggregation was monitored at 37°C using a Born dual-channel aggregometer (Labor, Hamburg, Germany) as described previously [25]. The aggregometer was adjusted before each test so that in each subject the value for light transmission for PRP was 0% and that for PPP was 100%. Aggregation was induced in duplicate using final concentrations of 2–20 μM adenosine diphosphate (ADP), and was monitored for 3 min. As we had previously observed that L-arginine decreased both the maximal extent and the maximal gradient of the aggregation curve, aggregations were evaluated as area under the aggregation curves [25].

2.3. Determination of urinary prostanoid and nitrate excretion rates

Urinary samples were kept at −20°C until analysis of their prostanoid content. Quantification of urinary 2,3-di-

nor-TXB_2 and 2,3-dinor-6-keto-$PGF_{1\alpha}$ was performed by negative chemical ionization gas chromatography–tandem mass spectrometry (GC–MS/MS) on a triple stage quadrupole mass spectrometer TSQ 45 (Finnigan MAT, San Jose, CA, USA) as described elsewhere [19]. Briefly, endogenous prostanoids and their corresponding tetradeuterated internal standards, which had been externally added to 50 ml aliquots of urine samples, were extracted from acidified urine samples (pH 3.0) by solid-phase extraction on octadecyl silica cartridges (J.T. Baker, Deventer, The Netherlands). After derivatization to their pentafluorobenzyl ester methoxyamine derivatives and separation by reversed-phase HPLC the analytes were converted to their trimethylsilyl ether derivatives. GC–MS/MS was performed by selected reaction monitoring of the characteristic daughter ions generated by collision-activated dissociation of the corresponding parent ions for endogenous prostanoids and their stable-isotope labelled analogues.

Urinary nitrate excretion was determined by GC–MS using the pentafluorobenzyl-(PFB-) derivative of nitrate as described previously [22,26], using $[^{15}N]$-NO_3^- (MSD Isotopes Merck Frosst, Montreal, Canada) as internal standard. Quantitation was performed by selected ion monitoring at m/z 46 for endogenous NO_2^-/NO_3^- and m/z 47 for the internal standard. The detection limit of the method was 20 fmol nitrate. Intra-assay variability was below 3.8%.

Urinary creatinine was determined spectrophotometrically by the alkaline picric acid reaction with an automatic analyzer (Beckman, Galway, Ireland). The excretion rates of prostanoid metabolites and of nitrate were corrected by urinary creatinine concentration, to exclude changes due to variability in renal excretory function, as described previously [18,19,21].

2.4. Determination of plasma arginine and cholesterol

Plasma L-arginine concentrations were determined by HPLC using pre-column derivatization with *o*-phthalaldehyde (OPA) as described previously [27]. Prior to analysis, lipids were extracted from plasma samples with trifluoroethane (Merck, Darmstadt, Germany). The aqueous phase of the extracted plasma samples, and internal standards were extracted on CBA solid phase extraction cartridges (Varian, Harbor City, CA, USA), and incubated for exactly 30 s with the OPA reagent (5.4 mg/ml OPA in borate buffer, pH 8.5, containing 0.4% 2-mercaptoethanol) before automatic injection into the HPLC. Chromatographic separation was performed on a C_6H_5 column (Machercy and Nagel, Düren, Germany) with the fluorescence monitor set at $\lambda^{ex} = 340$ nm and $\lambda^{em} = 455$ nm. Samples were eluted from the column with 0.96% citric acid/methanol 2:1, pH 6.8, at a flow rate of 1 ml/min. The intra- and inter-assay variability of the method was 5.2% and 5.5%, respectively; the detection limit 0.1 μmol/l.

Plasma total cholesterol was determined by a commercial immunofluorescence assay system (TDX; Abbott Diagnostics, Wiesbaden, Germany).

2.5. Organ bath studies

The aortas were dissected free of adhering fat and connective tissue and placed into organ baths filled with oxygenated (95% O_2, 5% CO_2) modified Krebs solution (37°C, pH 7.4) of the following composition (in mM): Na^+ 145.0, K^+ 5.95, Ca^{2+} 1.7, Mg^{2+} 1.2, Cl^- 128.15, HCO_3^- 25.0, $H_2PO_4^-$ 1.2, SO_4^{2-} 1.2, glucose 10.6, EDTA 0.025, within 1 h of death. The vascular preparations were connected to force transducers for isometric tension recording. For 60 min the rings were gradually stretched to a resting tension of 2 g (which had previously been determined to be the optimum of their length–tension relation), and repeatedly washed with fresh Krebs solution. The rings were then contracted with noradrenaline (1 μM) and relaxed by acetylcholine (1 μM) for testing of endothelial integrity as described previously [20]. Rings from control animals always showed relaxations greater than 70% of the noradrenaline-induced contraction plateau. After wash-out, cumulative concentration response curves were obtained with the endothelium-dependent relaxant ADP and the endothelium-independent relaxant sodium nitroprusside after precontraction with 1 μM noradrenaline, and the vasoconstrictors noradrenaline and serotonin (all drugs 1 nM to 0.1 mM). Relaxations were expressed as per cent of the precontractile tension induced with 1 μM noradrenaline. Noradrenaline, sodium nitroprusside, acetylcholine, and serotonin were purchased from Sigma (Munich, Germany). ADP was purchased from Boehringer (Mannheim, Germany).

2.6. Aortic atherosclerotic plaque formation

Segments of the thoracic aorta immediately distal from the left subclavian artery were fixed in formalin, embedded in paraffin, and stained with haematoxylin/eosin for the morphometric measurement of intimal and medial cross-sectional areas by planimetry using a semiautomatic system (Zeiss). Four sections of each animal were analyzed, and the values were averaged.

2.7. Calculations and statistics

All values are given as mean ± S.E.M. Statistical significance was tested using analysis of variance for repeated measures followed by the Scheffé *f*-test. For statistical comparison of the time course of urinary prostanoid excretion and of urinary nitrate excretion, the area under the curve (AUC) was calculated for each group, and AUC values were compared using Student's unpaired *t*-test. EC_{50} values (concentrations of drugs inducing half-maximal responses) for the organ bath experiments were calculated according to the method of Hafner et al. [28], and

compared using ANOVA followed by Fisher's protected least-significant-difference test. Statistical significance was accepted at the 0.05 level of probability.

3. Results

3.1. Urinary prostanoid excretion

Basal urinary 2,3-dinor-TXB_2 excretion was 4214.8 ± 275.5 pg/mg creatinine with no significant differences between the three groups. In the control group, 2,3-dinor-TXB_2 excretion decreased over time, reaching 1521.6 ± 323.6 pg/mg creatinine in the 12th week. Cholesterol feeding significantly increased urinary 2,3-dinor-TXB_2 excretion by 40–60% as compared to the control group during the whole study period ($p < 0.05$ vs. control; Fig. 1a). Supplementation with dietary L-arginine significantly decreased 2,3-dinor-TXB_2 excretion to 15–30% below control levels for most of the experimental period (AUC: $p < 0.05$ vs. control and $p < 0.01$ vs. cholesterol).

Urinary 2,3-dinor-6-keto-$PGF_{1\alpha}$ excretion, which was 4830.0 ± 205.9 pg/mg creatinine with no significant differences between the groups at baseline, also decreased in the control group during the study period (to 845.4 ± 178.7 pg/mg creatinine in week 12). In cholesterol-fed rabbits, 2,3-dinor-6-keto-$PGF_{1\alpha}$ excretion initially increased by 60 ± 15% ($p < 0.05$; Fig. 1b), but starting in week 3, excretion rates returned to levels not significantly different from the control group. Dietary L-arginine inhibited the early increase in 2,3-dinor-6-keto-$PGF_{1\alpha}$ excretion ($p < 0.05$ vs. cholesterol). 2,3-dinor-6-keto-$PGF_{1\alpha}$ values in this group were not significantly different from those in the control group.

The ratio of 2,3-dinor-6-keto-$PGF_{1\alpha}$/2,3-dinor-TXB_2 was 1.2 ± 0.1 at baseline and remained largely unchanged in the control group throughout the experimental period (Fig. 1c). It was not significantly changed in the cholesterol-fed group. In rabbits given cholesterol + L-arginine, the ratio was highly elevated until the 5th week and then returned to control levels (AUC: $p < 0.05$ vs. control and $p < 0.01$ vs. cholesterol).

3.2. Urinary nitrate excretion

Baseline urinary nitrate excretion was 955.5 ± 49.2 μmol/mmol creatinine. After 12 weeks, urinary nitrate excretion was 1053.8 ± 54.0 in the control group (p = n.s. vs. baseline). Urinary nitrate excretion decreased in the cholesterol-fed group during the first 6 weeks of dietary intervention, to arrive at a plateau about 50% below baseline and controls during the second half of the study period (AUC: $p < 0.05$ vs. control; Fig. 2). Dietary L-arginine reduced the extent of this decrease in urinary nitrate excretion by about 50%, but did not completely prevent it (AUC: $p < 0.05$ vs. control, $p < 0.05$ vs. cholesterol). In

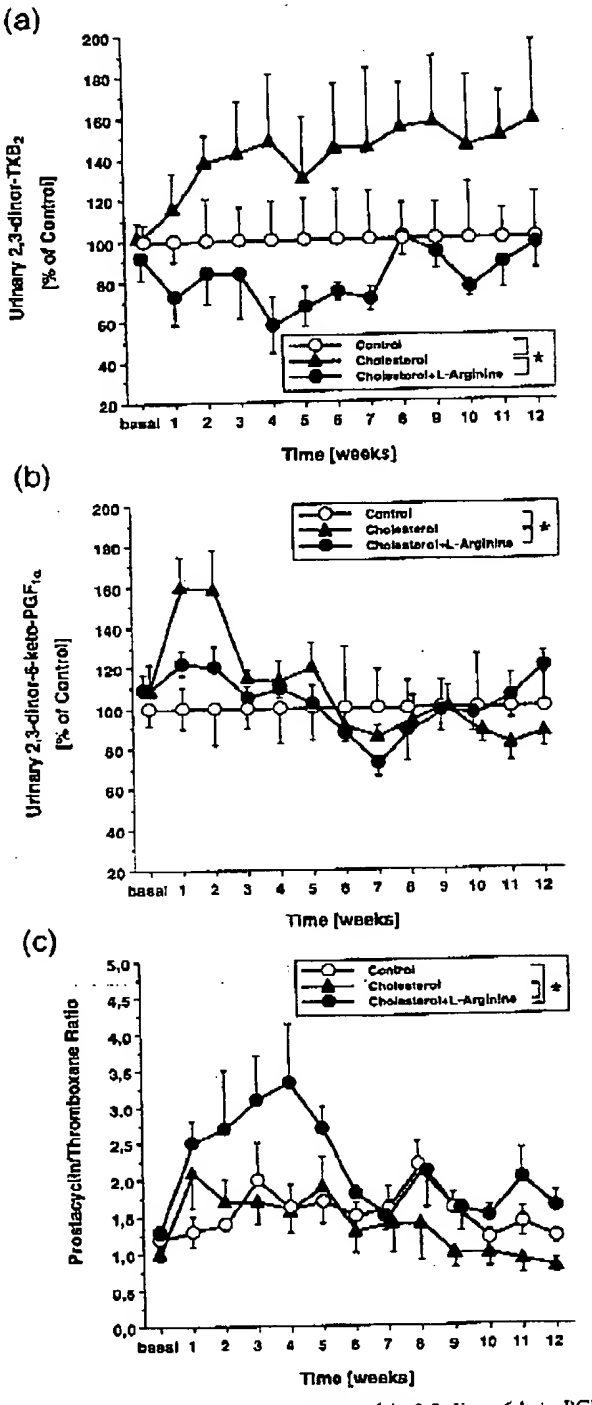

Fig. 1. Urinary 2,3-dinor-TXB_2 excretion (a), 2,3-dinor-6-keto-$PGF_{1\alpha}$ excretion (b), and ratio of urinary 2,3-dinor-6-keto-$PGF_{1\alpha}$/2,3-dinor-TXB_2 (c) in 24-h urines from rabbits during 12 weeks of dietary intervention. Values are means ± S.E.M. For statistical comparison between the groups, areas under the curves were calculated from the 1st through 12th weeks and compared using ANOVA followed by Fisher's protected least-significant-difference test. * $P < 0.05$ vs. cholesterol.

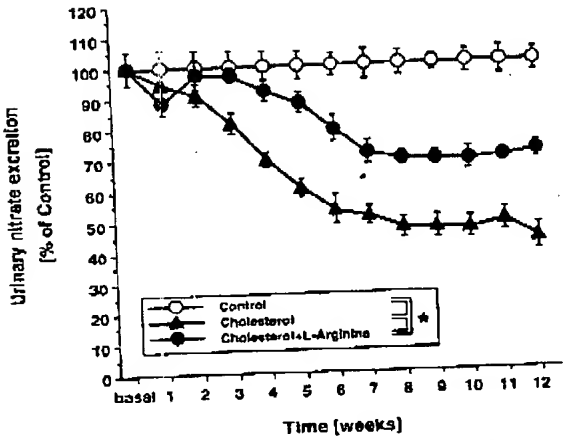

Fig. 2. Urinary nitrate excretion in 24-h urines from rabbits during 12 weeks of dietary intervention. Values are means ± S.E.M. For statistical comparison between the groups, areas under the curves were calculated from the 1st through 12th weeks and compared using ANOVA followed by Fisher's protected least-significant-difference test. $^{*}P < 0.05$ vs. cholesterol.

multiple correlation analyses, the reduction in urinary nitrate excretion rates was correlated with the increase in urinary 2,3-dinor-TXB_2 excretion in the cholesterol group ($R = 0.48$, $p < 0.01$). L-arginine supplementation abolished this linear relationship ($R = 0.08$; p = n.s.). There was no significant correlation between nitrate excretion, 2,3-dinor-6-keto-$PGF_{1\alpha}$ excretion, platelet aggregation and total cholesterol concentrations.

3.3. Platelet aggregation

Platelet aggregation in response to ADP was significantly increased in hypercholesterolaemic rabbits as compared to controls over the whole concentration range of ADP ($p < 0.05$; Fig. 3). Dietary L-arginine inhibited both the maximal extent and the maximal slope of the aggregation curves. The area under the aggregation curves was decreased to a level slightly below, but not statistically significantly different from the control group ($p < 0.05$).

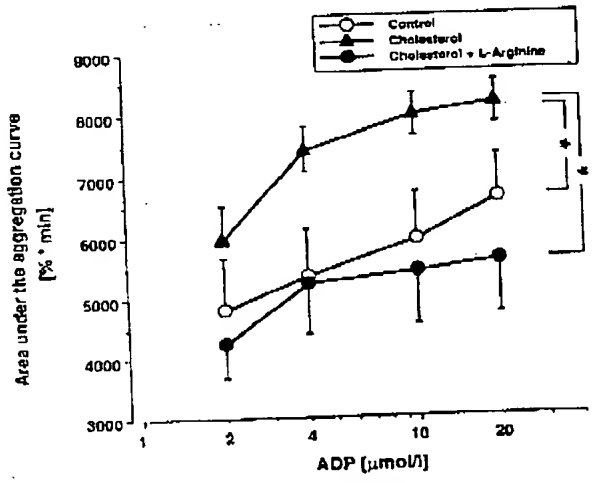

Fig. 3. Platelet aggregation induced by ADP (10 µM) in platelet-rich plasma ex vivo from rabbits fed a normal diet (Control), a diet enriched with 1% cholesterol (Cholesterol), or 1% cholesterol + 2.25% L-arginine in drinking water (Cholesterol + L-arginine). $^{*}P < 0.05$ vs. control. $^{\dagger}P < 0.05$ vs. cholesterol.

3.4. Vascular reactivity

Endothelium-dependent relaxations in response to ADP were significantly attenuated in the cholesterol-fed rabbits as compared to controls (Fig. 4a). Dietary L-arginine partly, but not completely restored relaxations to ADP ($p < 0.05$ vs. cholesterol). In contrast, no significant differences were observed in endothelium-independent relaxations in response to sodium nitroprusside between all three groups (Fig. 4b).

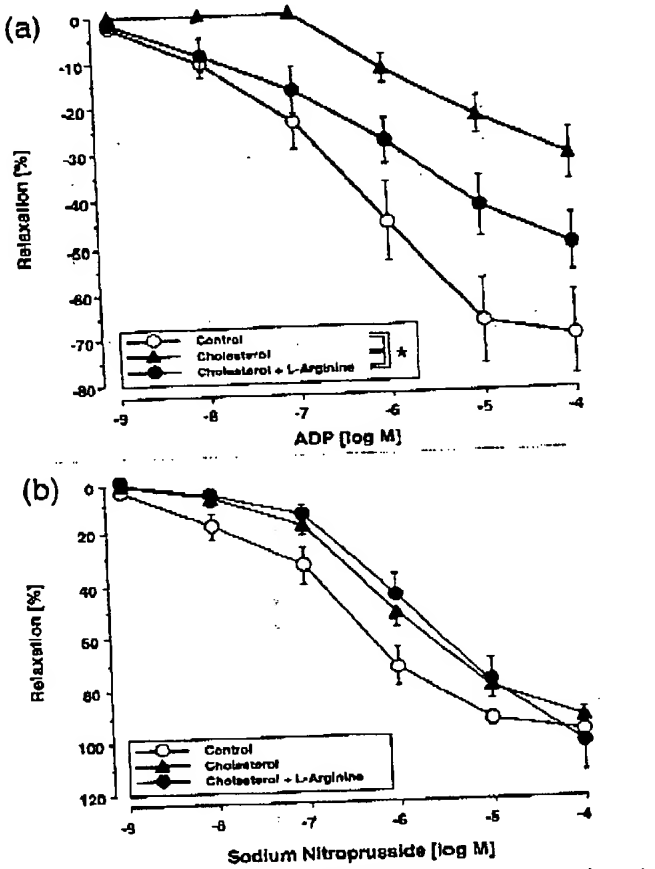

Fig. 4. Endothelium-dependent relaxations induced by ADP (a), and endothelium-independent relaxations induced by sodium nitroprusside (b) of isolated aortic rings ex vivo from rabbits fed normal rabbit chow (control), or a diet enriched with 1% cholesterol (cholesterol), or a cholesterol-enriched diet plus L-arginine (L-arginine) in drinking water for 12 weeks. Values represent means ± S.E.M. of 4 rings from 8 rabbits per group (4 rabbits in the control group).

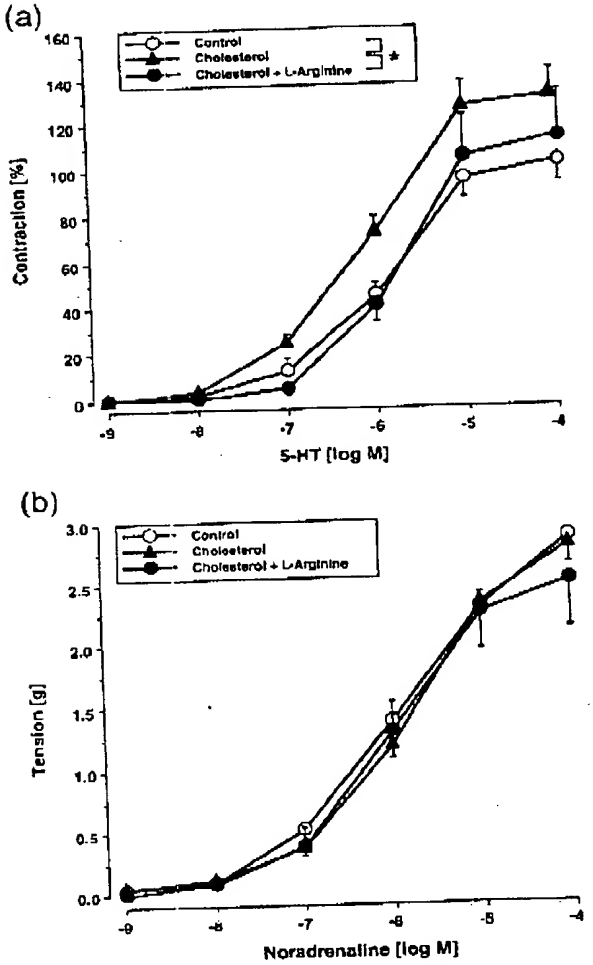

Fig. 5. Endothelium-dependent contractions induced by serotonin (a), and endothelium-independent contractions induced by noradrenaline (b) of isolated aortic rings ex vivo from rabbits fed normal rabbit chow (control), or a diet enriched with 1% cholesterol (cholesterol), or a cholesterol-enriched diet plus L-arginine (L-arginine) in drinking water for 12 weeks. Values represent means ± S.E.M. of 4 rings from 8 rabbits per group (4 rabbits in the control group).

Table 1
EC_{50} values for endothelium-dependent and -independent vasoactive mediators tested in vitro

	(−log EC_{50})			
	ADP	SNP	5-HT	NA
Control	6.79 ± 0.23*	5.51 ± 0.29	5.69 ± 0.60*	6.01 ± 0.14
Cholesterol	5.22 ± 0.12	5.67 ± 0.15	6.00 ± 0.68	5.69 ± 0.86
Cholesterol + L-arginine	6.13 ± 0.22*	5.50 ± 0.16	5.65 ± 0.72*	5.66 ± 0.74

Values are mean ± S.E.M.; * $p < 0.05$ vs. cholesterol.
Abbreviations: ADP, adenosine diphosphate; SNP, sodium nitroprusside; 5-HT, 5-hydroxytryptamine (serotonin); NA, noradrenaline.

Table 2
Plasma L-arginine and cholesterol concentrations

	Group	Baseline	12 weeks
L-arginine (μmol/l)	Control	88.3 ± 11.6	82.3 ± 14.7
	Cholesterol	87.0 ± 24.2	85.1 ± 11.5
	Chol + L-arginine	87.8 ± 14.6	217.3 ± 25.3*
Cholesterol (μmol/l)	Control	0.72 ± 0.08	0.65 ± 0.12
	Cholesterol	0.74 ± 0.08	20.70 ± 1.35*
	Chol + L-arginine	0.79 ± 0.09	20.87 ± 2.01*

Values are mean ± S.E.M.; * $p < 0.05$ vs. baseline.

Contractions in response to serotonin were significantly enhanced in the cholesterol-fed group as compared to controls (Fig. 5a). L-arginine resulted in a reduction of the contractile effect of serotonin, which was not significantly different between the L-arginine-treated and the control group. In contrast, noradrenaline contractions were not significantly different between the groups (Fig. 5b). The EC_{50} values for the endothelium-dependent and -independent vasoconstrictors and vasodilators are given in Table 1.

3.5. Aortic plaque formation

In aortic cross-sections from control rabbits, only the endothelial monolayer was visible with no intimal plaques (intima/media ratio, 0). In cholesterol-fed rabbits significant intimal thickening was observed. Intima/media ratio in this group was calculated to be 1.9 ± 0.3. Chronic dietary supplementation with L-arginine significantly reduced intimal atherosclerotic plaque formation (intima/media ratio, 0.7 ± 0.2; $p < 0.05$ vs. cholesterol).

3.6. Plasma cholesterol and L-arginine levels

Plasma total cholesterol was 0.75 ± 0.04 mmol/l at baseline and was not significantly changed in the control group during the study period. It increased to 20.87 ± 0.86 mmol/l in the rabbits fed a cholesterol-enriched diet. Dietary L-arginine had no effect on plasma cholesterol concentrations (Table 2).

Plasma L-arginine concentration was 87.2 ± 8.8 μmol/l at baseline and remained unchanged in the control group. Cholesterol feeding alone did not affect plasma L-arginine concentrations, but dietary L-arginine resulted in an about threefold increase in plasma L-arginine concentrations (Table 2).

4. Discussion

Our present study suggests that hypercholesterolaemia increases thromboxane A_2 synthesis in vivo in cholesterol-fed rabbits. This is paralleled by decreased systemic NO formation, as assessed by measuring the urinary excretion rate of nitrate, the oxidative metabolite of NO, and by platelet hyperactivity. In contrast, prostacyclin synthesis is

increased in early hypercholesterolaemia and reduced in later phases of atherogenesis. Dietary L-arginine partly prevents both the decrease in NO synthesis and the elevated thromboxane formation in vivo; it inhibits platelet aggregation ex vivo in response to ADP. Furthermore, endothelium-dependent vascular responses to ADP and serotonin, two potent platelet-derived modulators of vascular tone, which are abnormal in hypercholesterolaemia, are restored by L-arginine.

Platelet activity has previously been shown to be enhanced in hypercholesterolaemic rabbits [29,30] and in human hypercholesterolaemia and atherosclerosis [7,8]. Different mechanisms may contribute to this increased platelet activity in hypercholesterolaemia: Changes in lipid content of platelet membranes may facilitate their activation by physiological stimuli [31]. Moreover, superoxide anions released by polymorphonuclear leukocytes have been shown to activate platelets [32]. Superoxide radical production is enhanced in the hypercholesterolaemic vascular wall [20,24] and may, among other effects, contribute to increased platelet activity. The main underlying cause of platelet activation and endothelial dysfunction in this animal model of atherosclerosis is decreased NO elaboration in the vascular endothelium [20,23,30]. Our present results show that the urinary excretion of nitrate is progressively decreased in hypercholesterolaemic rabbits, and NO-dependent relaxations to ADP are impaired, pointing to a progressive deterioration of endothelial NO elaboration during the course of cholesterol feeding. These data are in line with similar observations made by ourselves and others with different endothelium-dependent mediators of vasorelaxation [1,2,20]. Furthermore, the finding that dietary supplementation with L-arginine, which restores NO elaboration by the endothelium, inhibits platelet aggregation and thromboxane formation, also indicates that defective activity of NO is the underlying cause for the close interplay between endothelial cell and platelet function in hypercholesterolaemic rabbits. Kaul et al. [11] also demonstrated that atherosclerosis markedly impaired vasodilator responses of perfused rabbit carotid arteries in response to activated human platelets in vitro, probably because vasodilation to ADP released from platelets was impaired due to the NO-deficient, atherosclerotic endothelium. Factors like endothelial platelet adhesion and interactions of platelets with endothelial cells and other cell types may therefore contribute to modulating platelet activity in vivo [33].

Our present data corroborate the finding by Tsao et al. [30] that dietary supplementation with L-arginine reduced aortic intimal plaque formation and platelet activity despite continuously elevated cholesterol levels. Dietary L-arginine was therefore able to profoundly modify factors which are usually accepted as being implicated in atherosclerosis [10]. It may be speculated that differences in L-arginine content in various diets may contribute to the beneficial cardiovascular effects of vegetable/fish diets [34].

Treatment with the antiplatelet agent ticlopidine has also been shown to reduce platelet aggregation in cholesterol-fed rabbits [35], but its antiplatelet effect was attenuated in hypercholesterolaemia as compared to normocholesterolemic control rabbits. Moreover, ticlopidine even increased platelet thromboxane formation in response to ADP. By contrast, L-arginine treatment inhibited both platelet hyperactivity and thromboxane metabolite excretion in vivo in the present study. Therefore, the effects of L-arginine on platelet reactivity and thromboxane formation may be superior to established antiplatelet agents like ticlopidine in hypercholesterolaemia.

The balance between prostacyclin and thromboxane A_2 is suggested to be one of the major regulatory mechanisms for platelet activation in vivo [16]. However, radioimmunoassay determinations of the primary metabolites of prostacyclin and TXA_2 formed by spontaneous hydrolysis, i.e. 6-keto-$PGF_{1\alpha}$ and TXB_2, have yielded controversial results. Using these methods, it has been shown that slices of aortas from cholesterol-fed rabbits produced significantly less prostacyclin than controls [3]. Biopsy specimens of atherosclerotic human arteries have a reduced capacity to generate prostacyclin in vitro [36]. By contrast, Norman and Miller [12] found no correlation between plasma 6-keto-$PGF_{1\alpha}$ or TXB_2 levels and the progression of coronary atherosclerosis in hypercholesterolaemic swine. Mehta et al. [37] demonstrated that the conversion of [^{14}C]-arachidonic acid to 6-keto-[^{14}C]-$PGF_{1\alpha}$ and [^{14}C]-TXB_2 was increased in aortic segments from hypercholesterolaemic rabbits as compared to non-atherosclerotic segments.

The method of choice to reliably assess prostanoid production rates in vivo is the quantification of their specific, enzymatically formed urinary metabolites, i.e., 2,3-dinor-6-keto-$PGF_{1\alpha}$ for prostacyclin and 2,3-dinor-TXB_2 for TXA_2 [17]. This approach avoids sampling artifacts which may occur by vascular injury during the blood sampling procedure when plasma metabolites are measured, and represents a reliable method to assess the synthesis rates of these prostanoids in vivo [38]. Using this non-invasive approach, we have demonstrated for the first time in the present study that the time course of the activation of TXA_2 and prostacyclin formation in hypercholesterolemic rabbits differs: TXA_2 formation remained elevated throughout the experimental period, whereas prostacyclin production initially increased, but rapidly returned to control levels during the progression of atherosclerosis. Moreover, we showed that dietary supplementation with L-arginine not only inhibited platelet aggregation ex vivo and 2,3-dinor-TXB_2 excretion in vivo, by also prevented the early increase in 2,3-dinor-6-keto-$PGF_{1\alpha}$ excretion.

These phenomena may be explained by the observation that prostacyclin is synthesized in part from platelet-derived endoperoxide precursors, which are shifted from activated platelets to the endothelium [13–15]. Differential

activation of this endoperoxide shift in different stages of atherogenesis — and its inhibition by dietary L-arginine — may explain the time-dependent changes in prostanoid metabolite excretion in the present study. In early hypercholesterolaemia, platelet activation at the hypercholesterolaemic endothelium may occur secondarily to impaired NO activity. This results in enhanced release of TXA_2 and endoperoxide precursors from platelets, as shown by elevated urinary 2,3-dinor-TXB_2 excretion. Endoperoxides may then be taken up by endothelial cells, where they can be utilized as a substrate for prostacyclin synthesis. This results in a transiently increased prostacyclin synthesis, as demonstrated by elevated urinary 2,3-dinor-6-keto-$PGF_{1\alpha}$ excretion. Later on in the atherosclerotic process, aggravation of endothelial dysfunction may result in decreased prostacyclin biosynthetic capacity, resulting in normalized prostacyclin formation rates in spite of continuously elevated TXA_2 formation. Inhibition of platelet activity and TXA_2 formation by dietary L-arginine also attenuated PGI_2 synthesis in the first half of the study via reduced endoperoxide shift. These differences in the time course of the formation of these two prostanoids may explain in part the differential changes of vascular reactivity observed by others in early hypercholesterolaemia and advanced atherosclerosis [39], and the controversial results found in in vitro studies [3,12,36,37].

Other than platelet sources of TXA_2 may contribute to its increased synthesis: It is well known that about 10–20% of 2,3-dinor-TXB_2 excreted into the urine is derived from non-platelet sources [17]. Cultured endothelial cells have been shown to release a substance with thromboxane-like immunoreactivity after incubation with LDL cholesterol in vitro [40]. Moreover, other vasoconstrictor mediators released by platelets and other cellular sources may also contribute to enhanced vasoconstrictor tone in hypercholesterolaemia. The effect of hypercholesterolaemia on serotonergic vasoconstriction is of particular interest, since 5-HT together with TXA_2 is an important mediator of coronary vasoconstriction in response to platelet aggregation [9,41], and rabbit platelets contain more 5-HT than human platelets [42]. Hypercholesterolaemia enhances serotonergic vasoconstriction in conductance arteries [43,44] and resistance vessels [45]. Enhanced vasoconstrictor responses to 5-HT in hypercholesterolaemic porcine coronary arteries have been linked to a reduced basal or 5-HT-induced release of NO from the endothelium [44,46]. Therefore, the enhanced vasoconstriction in response to 5-HT and its normalization by dietary L-arginine in our present study is indicative of decreased NO formation in the hypercholesterolaemic endothelium and its restoration by chronic dietary L-arginine.

In conclusion, our present study shows that the balance of prostacyclin and TXA_2 is disturbed in hypercholesterolaemic rabbits in vivo. In early hypercholesterolaemia, prostacyclin formation is stimulated and may counteract platelet activation, but later on in the atherogenetic process, TXA_2 production outweighs prostacyclin formation. This is paralleled by enhanced vasoconstriction in response to serotonin and impaired vasodilation to ADP. Dietary supplementation with L-arginine prevents these changes in prostanoid balance, improves endothelium-mediated vascular responses ex vivo, and enhances urinary nitrate excretion in vivo. Platelet-derived mediators may significantly contribute to the progression of atherosclerosis in hypercholesterolaemic rabbits, and the inhibitory effect of L-arginine on platelet activity may at least partly explain its anti-atherosclerotic effects in this model.

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Dose-related effect of intravenous L-arginine on muscular blood flow of the calf in patients with peripheral vascular disease: a $H_2{}^{15}O$ positron emission tomography study

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1. Endothelium-derived nitric oxide (NO) contributes to the regulation of vascular tone and blood pressure. Infusion of L-arginine produces systemic vasodilatation via stimulation of endogenous NO formation. Vasodilatation is accompanied by an increase in peripheral arterial blood flow. However, it is not known whether capillary nutritive blood flow increases as well. The time course and dose–response pattern of this effect remain to be elucidated.

2. Two groups of ten patients with peripheral vascular disease (PVD) received an intravenous infusion of 8 g or 30 g of L-arginine over a period of 40 min. Blood pressure and heart rate were monitored non-invasively. Muscular blood flow (MBF) of the calf was determined at 0, 20, 40, 60, 80 min by positron emission tomography with $H_2{}^{15}O$ as flow tracer. Plasma L-arginine and cyclic GMP (cGMP) levels were determined at the same time points.

3. L-arginine induced a dose-related decrease in blood pressure during the infusion period. MBF and plasma cGMP levels during and after the infusion of 8 g of L-arginine did not change significantly. In the patients receiving 30 g of L-arginine, MBF was enhanced significantly from 1.56 ± 0.14 to 2.09 ± 0.21 ml min^{-1} 100 ml^{-1} at 40 min and 2.23 ± 0.15 ml min^{-1} 100 ml^{-1} after 80 min ($+43.0\%$). The increase in MBF was paralleled by an increase in plasma cGMP from 4789.8 ± 392.2 nmol/l at baseline to 9223.2 ± 1233.6 nmol/l at 40 min.

4. We conclude that intravenous L-arginine enhances nutritive capillary MBF in patients with PVD via the NO–cGMP pathway in a dose-related manner. This effect might be therapeutically beneficial in patients with PVD.

INTRODUCTION

Endothelium-derived nitric oxide (NO), which is synthesized from the terminal guanidino nitrogen of the amino acid precursor L-arginine [1], has been shown to account for the biological activity of endothelium-derived relaxing factor [2]. These actions, mainly relaxation of vascular smooth muscle cells and inhibition of platelet aggregation and adhesion, are mediated by the intracellular second messenger cyclic GMP (cGMP) [3]. Basal secretion of NO in healthy blood vessels has been shown to contribute to the regulation of blood pressure by inducing an active vasodilatory tone [4]. In hypercholesterolaemic and atherosclerotic blood vessels, endothelium-dependent vascular relaxation is well known to be impaired [5–8]. Exogenous administration of L-arginine, the precursor of endogenous NO, has been shown to enhance endothelial function and to reduce intimal plaque area in animal models of hypercholesterolaemia [8–10].

In healthy human subjects, intravenous L-arginine induces peripheral vasodilatation, inhibits platelet aggregation and concomitantly increases urinary NO_3^- and cGMP excretion rates [11]. L-Arginine has also been shown to enhance acetylcholine-induced, endothelium-dependent vasodilatation in hypercholesterolaemic or atherosclerotic patients [12, 13]. Moreover, we have recently demonstrated in a double-blind, controlled study that a single intravenous infusion of L-arginine induces peripheral vasodilatation in patients with critical limb ischaemia [14]. These results have to be further clarified in two respects. Firstly, the question arises whether the observed increase of blood flow is due to an increase of nutritive tissue perfusion or merely to an opening of arterio–venous (AV) shunts. Second, further information about the time course

Key words: L-arginine, cyclic GMP, nitric oxide, positron emission tomography, skeletal muscle.

Abbreviations: ANOVA, analysis of variance; AV, arterio–venous; cGMP, cyclic guanosine monophosphate; MBF, muscular blood flow; NO, nitric oxide; OPA, o-phthalaldehyde; PET, positron emission tomography; PVD, peripheral vascular disease; VOP, venous occlusion plethysmography.

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of the drug effect and about the dose/effect relationship are needed.

Non-invasive assessment of peripheral blood flow has mainly relied on two methods: venous occlusion plethysmography (VOP) and duplex ultrasound. Both methods are appropriate to estimate total blood flow to an extremity or to a segment of an extremity. However, neither method provides any information on nutritive capillary blood flow in a defined tissue compartment of an extremity. Moreover, lack of accuracy in each single recording requires repeated testing to generate one averaged value [15, 16].

Positron emission tomography (PET) is a well-established method to provide absolute values of nutritive capillary tissue flow in brain [17], heart [18] and tumours [19]. This is accomplished most often with the flow tracers NH_3 or H_2O. Recently, we have shown that $H_2{}^{15}O$-PET is applicable to the assessment of skeletal muscle blood flow as well, particularly in peripheral vascular disease (PVD) [20]. The peculiarities of skeletal muscle perfusion, i.e. marked low flow at rest and great flow reserve, require precautions in designing protocols for data acquisition, data processing and image analysis. Provided the design requirements are met, accurate results in terms of absolute flow values can be obtained. Thus, in the present study we investigated whether L-arginine, given as a single intravenous infusion in two different dosages, increases nutritive muscular blood flow of the calf in patients with peripheral vascular disease.

This work was presented at the 69th Scientific Sessions of the American Heart Association, New Orleans, 10–13 November 1996.

METHODS

Patients and study design

Twenty patients with PVD (Fontaine stages IIB–IV) confirmed by angiography were studied. The clinical characteristics of the patients are shown in Table 1. All patients gave written informed consent before participation in the study, which had been approved by the Hannover Medical School Ethics Committee.

Table 1. Clinical characteristics of patients participating in the study, including age and sex, severity of disease, and risk profiles

	L-Arginine (8 g)	L-Arginine (30 g)
No. of patients	10	10
Male (*n*)	6	8
Female (*n*)	4	2
Age (years ± SD)	61.1 ± 14.8	74.8 ± 6.2
Fontaine stage II (*n*)	8	8
Fontaine stage III (*n*)	1	2
Fontaine stage IV (*n*)	1	0
Hypertension (*n*)	4	7
History of smoking (*n*)	7	5
Diabetes mellitus (*n*)	2	2
Hypercholesterolaemia (*n*)	4	6

Patients were placed in the supine position with the maximum calf diameter in the middle of the field of view of the PET camera. Attention was paid to ensure that the legs rested without compression by the patient's bed. For arterial blood sampling the radial artery was cannulated. An intravenous line for application of the infusions was placed into the cubital vein of the contralateral side. Blood presssure and heart rate were measured non-invasively throughout the study by an automatic device at intervals of 5 min. After 30 min of rest, a baseline PET scan was performed. Then the patients received a single intravenous infusion of L-arginine (Braun; L-arginine/HCl dissolved in 150 ml of 0.9% saline, pH 6.5) in a dose of 8 g or 30 g over a period of 40 min. Follow-up PET scans were performed at 20, 40, 60 and 80 min after the start of the infusion. At the time of each PET scan, blood samples were drawn for the determination of plasma L-arginine and cGMP concentrations.

PET

Data acquisition, data processing and image analysis are described elsewhere in detail [20]. Briefly, flow studies of the calf were performed with a Siemens/CTI ECAT 951/31 PET scanner. Thirty-one slices with a plane separation of 3.4 mm were obtained simultaneously. After intravenous bolus injection of 1.85 GBq of $H_2{}^{15}O$ a dynamic scan was acquired within 5 min (frame rate 12×5 s, 4×15 s, 2×30 s, 2×60 s). All image data were corrected for attenuation (10 min transmission) and radioactive decay. Arterial blood samples were taken at mid-frame times.

Mathematical modelling of the measured parameters (arterial input activity concentration and tissue activity concentration) is based on the assumption that water is freely diffusible. Thus, tracer kinetics can be described by the Kety–Schmidt equation [21]. We applied a linearized modification of this equation as described by van den Hoff et al. [22] to generate parametric flow maps on a pixel by pixel basis. This equation allows correction for delay and dispersion of the arterial input function as well as for fractional blood volume. The final value denotes capillary nutritive tissue blood flow in ml/min per 100 ml of tissue.

Quantitative image analysis was performed on the average of nine central slices. Definition of regions of interest was based on anatomical structures of the leg. MBF was calculated from a region of interest which included all muscles in the cross-section of the calf. Tibial and fibular bones were excluded as were the three main arteries (Fig. 1). Separate flow values were obtained for both legs at each scan.

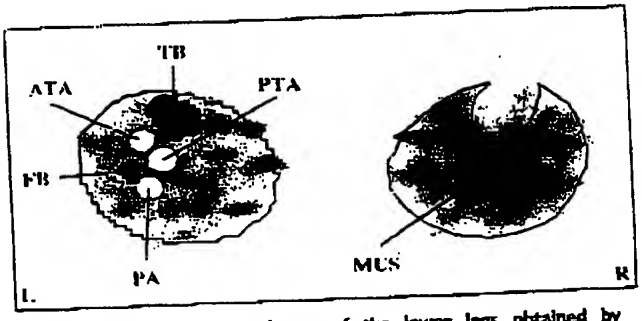

Fig. 1. Parametric flow image of the lower legs obtained by $H_2{}^{15}O$-PET. The bones had been identified on the transmission scan. The arteries had been identified on images showing the early, i.e. the arterial phase, of tracer distribution. MUS indicates the region of interest from which muscle blood flow values were computed. TB = tibial bone; FB = fibular bone; ATA = anterior tibial artery; PTA = posterior tibial artery; PA = peroneal artery.

Plasma L-arginine and cGMP concentrations

Plasma L-arginine concentrations were determined by HPLC using pre-column derivatization with *o*-phthalaldehyde (OPA) as described previously [22a]. Briefly, plasma samples were extracted on CBA solid phase extraction cartridges (Varian), dried over nitrogen and dissolved in doubly distilled water for HPLC analysis. HPLC was carried out on a Gynkotek liquid chromatography system consisting of two HPLC pumps with a gradient controller (model M 480 HDG), a spectral fluorescence detector (RF 1002), and an automatic injector (model GINA 160). Samples and standards were incubated for exactly 30 s with the OPA reagent (5.4 mg/ml OPA in borate buffer, pH 8.5, containing 0.4% 2-mercaptoethanol) before automatic injection into the HPLC system. Chromatographic separation was performed on a C6H5 column (Macherey and Nagel) with the fluorescence monitor set at λ excitation = 340 nm and λ emission = 455 nm. Samples were eluted from the column with 0.96% citric acid/methanol 2:1, pH 6.8, at a flow rate of 1 ml/min. The coefficient of variation of the method had previously been determined as 5.2% within-assay and 5.5% between-assay; the detection limit of the assay was 0.1 μmol/l.

For the determination of cGMP, plasma samples were thawed and centrifuged at 2500 *g* (4°C; 10 min). Supernatants were diluted 1:4 in PBC and acetylated by a mixture of acetic acid anhydride/triethylamine (1:2, v/v). cGMP content was measured by RIA using ^{125}I-labelled cGMP as a tracer and globulin precipitation. The detection limit of the assay was 160 nmol/l.

Calculations and statistical analysis

All values are given as means ± SEM. Statistical comparison of paired observations was done by the Wilcoxon signed rank test. Unpaired observations were compared by the Mann–Whitney U-test. For comparison of plasma L-arginine levels, plasma cGMP concentrations and muscular blood flow values between the two treatment groups factorial analysis of variance (ANOVA) was performed. In all tests statistical significance was assumed for $P < 0.05$.

RESULTS

Clinical observations

Intravenous infusion of L-arginine was tolerated well in both dosages. Only one patient in the 30 g group complained of discomfort of the mouth, the tongue and the hands in the first minutes of infusion. However, no physical signs could be observed. The infusion was continued and the sensations subsided. In both treatment groups two patients asked to interrupt the study after the third PET scan due to lower back pain or restlessness of the legs.

Plasma L-arginine and cGMP concentrations

The mean baseline plasma L-arginine concentration was 101.3 ± 14.1 μmol/l. Infusion of 30 g of L-arginine increased plasma L-arginine concentration to 8963.5 ± 1400.4 μmol/l at the end of the 40 min infusion period. At the end of the infusion of 8 g of L-arginine, L-arginine plasma concentration was increased to 1367.7 ± 136.3 μmol/l. The plasma concentrations differed significantly between the two groups at 20, 40, 60 and 80 min. After the end of the infusions, plasma L-arginine concentrations began to decrease; however, baseline plasma levels were not reached within the 40 min post-infusion period (Fig. 2).

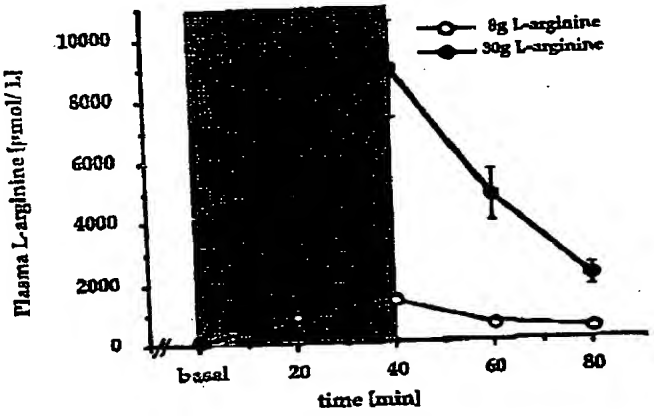

Fig. 2. Plot of plasma levels of L-arginine before (basal), during (20 min, 40 min) and after (60 min, 80 min) intravenous infusion of 8 g of L-arginine (open circles) and 30 g of L-arginine (closed circles) in patients with PVD. The shaded area indicates the infusion period. Values are given as means ± SEM of ten patients in each group. A P value of <0.0001 was found for comparison of the curves by factorial ANOVA.

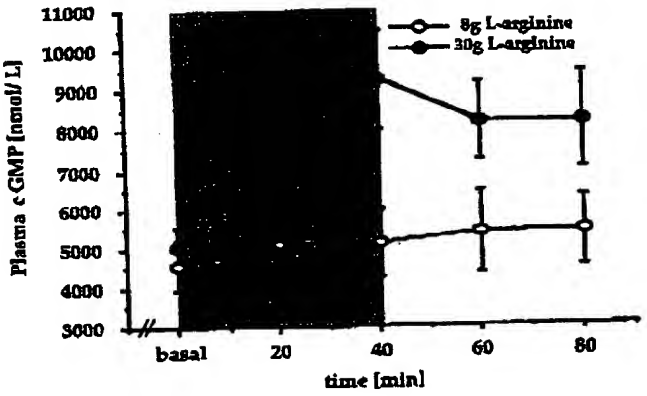

Fig. 3. Plot of plasma levels of cGMP before (basal), during (20 min, 40 min) and after (60 min, 80 min) intravenous infusion of 8 g of L-arginine (open circles) and 30 g of L-arginine (closed circles) in patients with PVD. The shaded area indicates the infusion period. Values are given as means ± SEM of ten patients in each group. A P value of <0.0001 was found for comparison of the curves by factorial ANOVA.

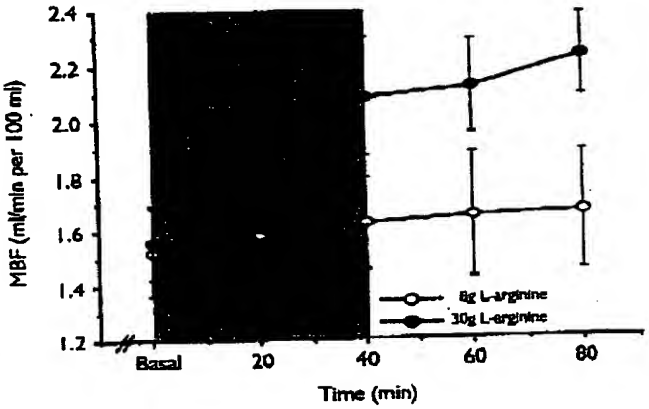

Fig. 4. Plot of MBF of the calfs before (basal), during (20 min, 40 min) and after (60 min, 80 min) intravenous infusion of 8 g of L-arginine (open circles) and 30 g of L-arginine (closed circles) in patients with PVD. The shaded area indicates the infusion period. Values are given as means ± SEM of ten patients in each group. A P value <0.05 was found for comparison of the curves by factorial ANOVA.

Plasma cGMP concentration was 4789.8 ± 392.2 nmol/l at baseline. During the infusion of 30 g of L-arginine it increased to 9223.2 ± 1233.6 nmol/l at 40 min (+83.8%; $P<0.05$ compared with baseline). After the end of the infusion, plasma cGMP decreased but did not reach baseline levels within the 40 min post-infusion period. During and after the infusion of 8 g of L-arginine, plasma cGMP only slightly increased by 12–18% (Fig. 3).

MBF

MBF is given as the mean value of the right and the left leg. No significant differences were observed between the legs at any time in any treatment group. Baseline values averaged 1.53 ± 0.16 ml/min per 100 ml in the 8 g group and 1.56 ± 0.14 ml/min per 100 ml of tissue in the 30 g group. In the 8 g group, MBF slightly increased during infusion of L-arginine, with a maximum of 1.67 ± 0.22 ml/min per 100 ml of tissue at 80 min (+9.0%). However, this increase was not statistically significant. In the 30 g group, the maximum value at 80 min was 2.23 ± 0.15 ml/min per 100 ml of tissue, denoting a relative flow increase of 43.0%. In this group, absolute flow values at 20, 40, 60 and 80 min differed statistically significantly from the baseline value, as did the corresponding 80 min values between the two treatment groups (Fig. 4).

Blood pressure and heart rate

Pre-infusion measurements of systolic blood pressure averaged 139.0 ± 6.6 mmHg in the 8 g group and 140.1 ± 4.2 mmHg in the 30 g group. Diastolic blood pressure values averaged 73.3 ± 2.4 and 71.1 ± 2.8 mmHg respectively. During the infusion period, systolic as well as diastolic blood pressure decreased in the 30 g group but not in the 8 g group (Fig. 5). In the 30 g group, the mean of all systolic measurements during infusion was 134.4 mmHg (−5.7 mmHg; $P<0.05$) and the diastolic value was 65.5 mmHg (−5.6 mmHg; $P<0.05$). In the 8 g group no significant changes could be detected. After the end of infusion, blood pressure increased rapidly in the 30 g group causing a significant overshoot. Neither of the doses of L-arginine had a significant effect on heart rate.

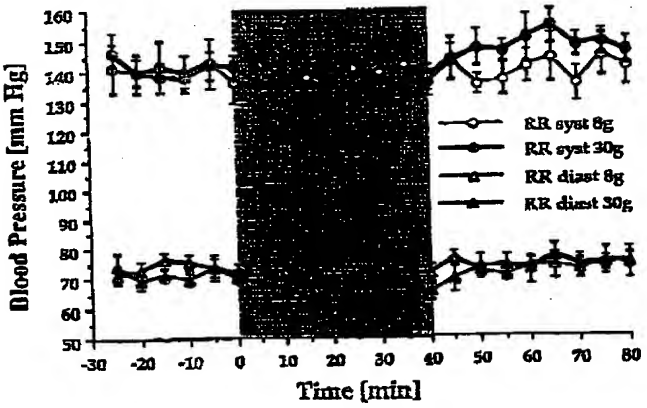

Fig. 5. Plot of systolic (circles) and diastolic (triangles) blood pressure [Riva-Rocci method (RR)] before, during and after intravenous infusion of 8 g of L-arginine (open plot symbols) and 30 g of L-arginine (closed plot symbols) in patients with PVD. The shaded area indicates the infusion period. Values are given as means ± SEM of ten patients in each group.

DISCUSSION

Our present study suggests that: (1) intravenous L-arginine increases nutritive MBF of the calf in patients with PVD via stimulation of the NO–cGMP pathway; (2) this effect of L-arginine on tissue perfusion is related to the dose; and (3) the increase in nutritive capillary flow persists when the blood pressure response to L-argininc has returned to baseline values.

Several studies have investigated the direct effects of L-arginine on peripheral blood flow in humans. Using VOP, Imaizumi et al. [23] demonstrated a dose-dependent increase in forearm blood flow in healthy humans by intra-arterial L-arginine in a dose range of 50–290 μmol/min. In this study D-arginine, in the same dose range, had no effect. Calver et al. [24] demonstrated by VOP that in a dose range from 10 to 40 μmol/min, neither L-arginine nor D-arginine had any vasodilatory effect in healthy humans. At a substantially higher dose (160 μmol/min), both L-and D-arginine induced vasodilatation in the present study. Panza et al. [25] also found no direct effect of intra-arterial L-arginine at an infusion rate of 40 μmol/min on forearm blood flow in hypercholesterolaemic patients, using the same method to assess forearm blood flow. However, they found an enhanced vasodilatory response to intra-arterial acetylcholine after L-arginine infusion.

Using intravenous infusions of L-arginine, Creager et al. [12] found no effect on forearm blood flow determined by VOP in patients with hypercholesterolaemia, nor in healthy human subjects, at an infusion rate of 3.3 mmol/min. However, we recently demonstrated by Duplex sonography that a single intravenous dose of L-arginine (4.75 mmol/min) increased femoral artery blood flow by 42.8% in patients with manifest atherosclerosis [14]. The conflicting results from these studies may be explained by the dose/effect relationships of L-arginine, with low doses having no direct vasodilatory effect on peripheral blood flow but enhancing acetylcholine-induced NO-dependent vasodilatation, medium doses inducing direct, NO-dependent vasodilatation, and with ultra-high doses of either L- or D-arginine inducing vasodilatation via unspecific mechanisms.

Up to now, no data has been available to demonstrate whether the blood flow response to L-arginine is due to an increased nutritive capillary blood flow or to an opening of AV shunts. Both reactions would result in increased blood flow velocity in the femoral artery, so that duplex ultrasound is inadequate to discriminate between these reactions. The same holds true for measurements by means of VOP. Furthermore, neither ultrasound nor VOP allow discrimination between cutaneous and muscular blood flow. In our present study, we applied $H_2{}^{15}O$-PET to further investigate this issue. The reproducibility and accuracy of this method in measuring tissue blood flow have been proven for the heart by using microspheres in a dog model [26]. The blood flow values in that study covered a range between 20 and 400 ml/min per 100 ml. As shown previously, this method is also applicable to skeletal muscle blood flow measurements within a flow range between 1 and 60 ml/min per 100 ml [20]. In thigh muscles, $H_2{}^{15}O$-PET has been used for the characterization of blood flow changes caused by hyperinsulinaemia [27]. In our present study, resting blood flow of patients with PVD did not differ from that previously measured in healthy subjects [28]. Intravenous infusion of L-arginine was followed by a marked increase in muscular blood flow after the 30 g dose (i.e. 4.75 mmol/min) whereas the 8 g dose (i.e. 1.27 mmol/min) induced virtually no reaction. The 43% flow increase in the 30 g group corresponds well to the flow increases determined by duplex ultrasound in healthy subjects (+44%) and in PVD patients (+43%) in our previous studies [11, 14]. This suggests that L-arginine obviously does not open AV shunts.

We have previously shown that nitrate, the oxidative metabolite of NO, and cGMP are useful markers to assess NO formation *in vivo* [11, 29, 30]. However, as plasma nitrate concentrations show large intra- and inter-individual variability, and urine sampling was not possible in the setting of PET scanning, we used plasma cGMP levels as a biochemical indicator of NO–cGMP pathway activation in the present study. There is broad evidence from studies *in vitro* that extracellular, i.e. plasma, cGMP levels represent the extent of intracellular NO induced signalling [31–33]. The haemodynamic changes induced by two different doses of intravenous L-arginine correlated well with the difference in plasma cGMP concentrations in both groups. These observations, together with the about 6-fold higher increase in plasma L-arginine concentration in the 30 g group, strongly suggest that the increase in nutritive muscular tissue blood flow was due to activation of the NO–cGMP pathway.

In addition to its impact on local tissue blood flow, intravenous L-arginine had an effect on systemic arterial blood pressure. Decreased systolic and diastolic blood pressure in response to intravenous L-arginine was first reported by Nakaki et al. [34]. Subsequently, this finding was confirmed, and evidence grew that it is NO-dependent [11, 35], although some authors could not reproduce this finding [36]. Our results also indicate that the effect of intravenous L-arginine on systemic arterial blood pressure is dose related.

In the present study, the time course of blood pressure was markedly different from that of tissue blood flow. Paralleled by the plasma cGMP levels, the increase in nutritive muscle blood flow outlasted the infusion period of L-arginine by at least 40 min (Figs. 3 and 4). A similar difference between the responses of (systemic) arterial pressure and (local) tissue blood flow has been noted in our previous study in patients with critical limb ischaemia [14]. In that study, femoral artery blood flow, mea-

sured by duplex ultrasound, continued to increase after the 60 min infusion period of 30 g of intravenous L-arginine and still remained elevated even 90 min after the infusion had been stopped. This observation would imply that L-arginine/NO acts in a different way on arteriolar resistance vessels (>25 μm in diameter), which contribute most to the regulation of peripheral vascular resistance, and on precapillary sphincter vessels (<25 μm in diameter), which control local tissue blood flow [37, 38]. The present PET study does not provide more detailed information to support this idea. However, our findings fit well with experimental data reported by Ekelund and Mellander [39] who demonstrated that the response of vascular resistance to intra-arterial administration of N^G-monomethyl-L-arginine followed by L-arginine was different between sections of 'large bore' resistance vessels and 'small-arteriolar' vessels in cat skeletal muscle.

In addition to the biological implications of our results, the study of nutritive capillary flow is significant in a clinical perspective. Patients with PVD might benefit from substances causing enhanced tissue blood flow in the leg muscles [40]. This is the mechanism by which substances like the prostaglandins are believed to act [41]. With its two main biological actions in man, increase of peripheral blood flow and inhibition of platelet aggregation [11], L-arginine has a pharmacological profile comparable with those compounds which are currently being used in the therapy of PVD. The fact that L-arginine not only increases peripheral blood flow in general but enhances nutritive capillary tissue blood flow provides a reason to set up clinical trials to study the possible therapeutic effects of L-arginine in PVD.

In conclusion, results from our present study indicate that the increase in arterial blood flow of the leg caused by intravenous L-arginine is due to increased nutritive capillary blood flow in skeletal muscle. This effect on local blood flow is most probably mediated by the NO–cGMP pathway. The increase in nutritive muscle blood flow by a well-tolerated intravenous dose of L-arginine might be beneficial in patients with PVD.

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Circulation

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Articles

L-Arginine Induces Nitric Oxide–Dependent Vasodilation in Patients With Critical Limb Ischemia

A Randomized, Controlled Study

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Abstract

Background L-Arginine is the precursor of endogenous nitric oxide (NO), which is a potent vasodilator acting via the intracellular second-messenger cGMP. In healthy humans, L-arginine induces peripheral vasodilation and inhibits platelet aggregation due to an increased NO production. Prostaglandin E_1 (PGE_1) induces peripheral vasodilation via stimulating prostacyclin receptors.

Methods and Results We investigated the effects of one intravenous infusion of L-arginine (30 g, 60 minutes) or PGE_1 (40 μg, 60 minutes) versus those of placebo (150 mL 0.9% saline, 60 minutes) on blood pressure, peripheral hemodynamics, and urinary NO_3^- and cGMP excretion rates in patients with critical limb ischemia (peripheral arterial occlusive disease stages Fontaine III or IV). Blood flow in the femoral artery was significantly increased by L-arginine (+42.3±7.9%, $P<.05$) and by PGE_1

(+31.0±10.2%, $P<.05$) but not by placebo (+4.3±13.0%, P=NS). Urinary NO_3^- excretion increased by 131.8±39.5% after L-arginine ($P<.05$) but only by 32.3±17.2% after PGE_1 (P=NS). Urinary cGMP excretion increased by 198.7±84.9% after L-arginine ($P<.05$) and by 94.2±58.8% after PGE_1 (P=NS). Both urinary index metabolites were unchanged by placebo.

Conclusions We conclude that intravenous L-arginine induces NO-dependent peripheral vasodilation in patients with critical limb ischemia. These effects are paralleled by increased urinary NO_3^- and cGMP excretion, indicating an enhanced systemic NO production. Increased urinary NO_3^- excretion may be a sum effect of NO synthase substrate provision (L-arginine) and increased shear stress (PGE_1 and L-arginine).

Key Words: L-arginine • prostaglandins • peripheral vascular disease • nitric oxide

Introduction

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The endothelium has been identified as a source of mediators that protect the vascular wall against vasospasm and thrombotic occlusion.[1] These mediators include prostacyclin, a vasodilator and antiaggregatory prostaglandin,[2] and nitric oxide (NO), which is synthesized from the terminal guanidino nitrogen of the amino acid precursor L-arginine.[3] NO has been shown to account for the biological activity of the endothelium-derived relaxing factor in the cardiovascular system.[4] These actions, mainly relaxation of vascular smooth muscle and inhibition of platelet aggregation and adhesion, are mediated by the intracellular second-messenger cGMP.[5] NO is very rapidly oxidized to NO_3^- in vivo,[6] which is subsequently excreted into the urine.[7] [8] As NO itself can hardly be measured in vivo, NO_3^- has been suggested to be a suitable index metabolite for the determination of NO formation rates in vivo.[8] [9] We have recently shown that intravenous L-arginine induces peripheral vasodilation, inhibits platelet aggregation, and concomitantly increases urinary NO_3^- and cGMP excretion rates in healthy humans.[10] The release and/or biological activity of endothelium-derived relaxing factor/NO has been shown to be impaired in atherosclerotic arteries,[11] [12] which is in accordance with the endothelial injury hypothesis of atherosclerosis.[13] Exogenous administration of L-arginine restores endothelium-dependent relaxations in experimental atherosclerosis.[14] [15] [16] However, although L-arginine was also shown to enhance acetylcholine-induced, endothelium-dependent vasodilation in hypercholesterolemic or atherosclerotic patients,[17] it has been disputed whether L-arginine is capable of inducing vasodilation in these patients.[18]

The biological activity of prostacyclin is also decreased in atherosclerosis, as the homeostasis between the vasoconstrictor thromboxane A_2 and prostacyclin is shifted in favor of thromboxane.[19] [20] Infusion of prostaglandin (PG)E_1, which stimulates prostacyclin receptors and thus substitutes its deficient biological activity, has been used as pharmacotherapy for peripheral arterial occlusive disease in Germany and several other countries.[21] [22]

In the present study, we investigated whether L-arginine, given as a single intravenous infusion, induces

vasodilation in the more severely affected lower limb of patients with critical limb ischemia and whether the possible hemodynamic effects are related to an increased NO production (using the urinary excretion rates of NO_3^- and cGMP as index parameters for systemic NO formation in vivo). We compared the effects of L-arginine with those of PGE_1 as an NO/cGMP-independent vasodilator and with those of placebo.

Methods

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Patients and Study Design

Ten male patients with critical limb ischemia (peripheral arterial occlusive disease stages Fontaine III or IV) received a single intravenous infusion of L-arginine (Fresenius AG; 30 g dissolved in 150 mL 0.9% saline, pH 6.5) or PGE_1 (Prostavasin, Schwarz Pharma; 40 μg dissolved in 150 mL 0.9% saline) into an antecubital vein over 60 minutes. Both substances were administered in randomized order with a washout period of at least 2 days between them. Another group of six patients with critical limb ischemia received a single intravenous infusion of placebo (150 mL 0.9% saline, 60 minutes). All patients had angiographically proven femoropopliteal occlusions and additional distal stenoses of the crural arteries. None had proximal hemodynamically relevant stenoses of the iliac vessels. The cardiovascular risk factors and parallel diseases of the two groups of patients are given in Table 1, and the mean plasma cholesterol and triglyceride levels are given in Table 2. Each patient gave written informed consent to participation in the study, which had been approved by the Hannover Medical School Ethics Committee. Comedication, which was kept constant throughout the study period, is given in Table 3. At the beginning of each study day, each patient emptied his bladder. A mild oral volume loading (using demineralized water) was started with 3 mL/kg body wt initially and continued during the study period with 1 to 2 $mL \cdot kg^{-1} \cdot h^{-1}$ adjusted to the individual hourly urine volumes. Each participant remained in the supine position for 60 minutes before and 30 minutes after the infusion.

View this table: [in this window] [in a new window] **Table 1.** Characteristics of Patients Participating in the Study, Including Fontaine Stage Classification of Peripheral Arterial Disease and Risk Profile/Parallel Diseases

View this table: [in this window] [in a new window] **Table 2.** Plasma Cholesterol and Triglyceride Levels

View this table: [in this window] [in a new window] **Table 3.** Comedications of Study Patients

During 1 hour before and 3 hours after the beginning of the infusion, urine was collected in hourly intervals and immediately frozen for the determination of urinary NO_3^- and cGMP concentrations. At baseline and at the end of the infusions, a venous blood sample was drawn with EDTA for the determination of plasma arginine levels.

At 60 minutes before, during, and for 20 minutes after the end of the infusion, blood pressure and heart rate were recorded every 5 minutes by the standard sphygomanometric method with an automatic device (Boso digital II, Bosch & Sohn).

Duplex Measurements of Femoral Arterial Blood Flow

Blood flow velocity was measured by image-directed duplex ultrasonography before the infusion and at its end in a segment of the common femoral artery with a circular cross section. Measurements were made with a DRF 400 image-directed duplex ultrasound system (Diasonics-Sonotron) with a transducer combining 7.5-MHz B-mode imaging and 3-MHz pulsed Doppler beams. Blood flow volume was automatically calculated as the product of the cross-sectional area and the time-averaged blood velocity from seven repeated measurements.[23] The investigator performing the duplex measurements was blinded to the treatment.

Biochemical Assays

Urinary NO_2^-/NO_3^- was determined as its pentafluorobenzyl derivative by gas chromatography–mass spectrometry (GC-MS) as described previously.[24] [25] Briefly, 100-μL aliquots of urine were spiked with 250 ng of $[^{15}N]NO_3^-$ (MSD Isotopes Merck Frosst) as internal standard, acidified with 20 μL of 0.1 N HCl, and treated with 5 mg cadmium for 10 minutes at room temperature. The suspension was then centrifuged; the supernatant decanted and alkalinized with 10 μL of 4 N NaOH, treated with 500 μL of cold acetone (-20°C), and centrifuged. Then, 5 μL of pentafluorobenzyl bromide was added to the decanted supernatant, and the mixture was allowed to react for 75 minutes at 50°C. After being cooled to room temperature, acetone was removed under nitrogen, and the residue was extracted with 1 mL of toluene. The toluene phase was taken up and dried over Na_2SO_4. Then 1-μL aliquots were injected into the GC-MS device.

GC-MS was carried out on a triple-stage quadrupole mass spectrometer TSQ 45 interfaced with a gas chromatograph 9611 (Finnigan MAT). An OV-1 fused silica capillary column (25x0.25 mm ID, 0.25-μm film thickness) from Machery-Nagel was used with helium as the carrier gas (55 kPa). Negative ions were produced by chemical ionization using methane as the reactant gas (65 Pa) at an electron energy of 90 eV and an electron current of 0.2 mA. Quantification was performed by selected ion monitoring at m/z 46 for endogenous NO_2^-/NO_3^- and m/z 47 for the internal standard. The detection limit of the method was 20 fmol nitrite or nitrate. Intra-assay variability was less than 3.8%.

For the determination of cGMP levels, urine samples were thawed and centrifuged at 2500*g* (4°C; 10 minutes). Supernatants were diluted 1:500 in phosphate buffered saline and acetylated by a mixture of acetic acid anhydride/triethylamine. cGMP content was measured by radioimmunoassay using $[^{125}I]$ cGMP as a tracer and globulin precipitation. The detection limit of the assay was 160 fmol/mL.

Urinary creatinine was determined spectrophotometrically with the alkaline picric acid method in an

automatic analyzer (Beckman). The urinary excretion rates of NO_3^- and cGMP were corrected by urinary creatinine concentration.

Plasma arginine levels were determined spectrophotometrically after conversion to urea according to the method of Bacchus and London.[26]

Calculations and Statistical Analysis

All values are given as mean±SEM. The statistical comparison of hemodynamic data was performed by two-way ANOVA for repeated measurements followed by the Scheffé F test. For statistical comparison of the time course of urinary NO_3^- and cGMP excretion, the area under the curve was calculated for each infusion, and area-under-the-curve values were compared using Student's paired *t* test. Statistical significance was assumed for $P<.05$.

Results

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Femoral arterial blood flow was enhanced by 42.3±7.9% during L-arginine infusion ($P<.05$) and by 31.0±10.2% during PGE_1 infusion ($P<.05$) but remained unchanged during placebo infusion (+4.3±13.0%, P=NS) (Fig 1). There was no significant difference between both active treatments. However, blood flow further increased until 30 minutes after the end of L-arginine infusion, whereas after the end of PGE_1 infusion blood flow immediately began to decrease. This effect was due to an increased blood flow velocity (+56% after L-arginine, +29% after PGE_1), whereas the femoral artery diameter remained unchanged (Table 4). Furthermore, L-arginine had a more pronounced effect on systolic and diastolic blood pressures than PGE_1, as assessed by comparison of the area under the blood pressure–time curves ($P<.05$), and placebo had no affect on blood pressure (Fig 2). Neither of the infusions significantly affected heart rates.

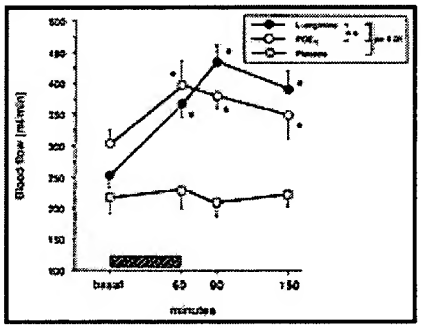

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Figure 1. Plot of blood flow in the superficial femoral artery determined by image-directed duplex sonography before (basal) and after the infusion of L-arginine (30 g, 60 minutes), PGE_1 (40 μg, 60 minutes), or placebo (150 mL 0.9% saline, 60 minutes) in patients with peripheral arterial occlusive disease. Values are mean±SEM of 10 patients (L-arginine, PGE_1) or 6 patients (placebo). *$P<.05$ vs baseline in multiple ANOVA. Striped bar indicates the duration of the infusions.

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Table 4. Femoral Artery Diameters and Blood Flow Velocities in Duplex Measurements at Baseline and After Infusions

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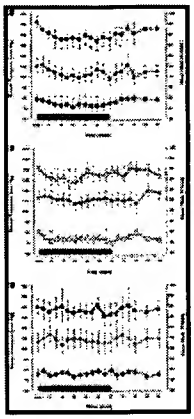

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Figure 2. Plots of time course of systolic (circles) and diastolic blood pressure (squares) and heart rate (triangles) in patients with peripheral arterial occlusive disease before, during, and after a 60-minute infusion of L-arginine (A), PGE_1 (B), or placebo (C). Values represent mean±SEM of 10 patients (L-arginine, PGE_1) or 6 patients (placebo). *P<.05 vs baseline in multiple ANOVA. Striped bar indicates the duration of the infusions.

During L-arginine administration, plasma arginine levels increased from 84.6±14.1 to 3780.3±380.5 μmol/L (P<.05). During the infusion of PGE_1 or placebo, plasma arginine levels were not significantly changed.

Urinary NO_3^- excretion significantly increased during L-arginine administration (+131.8±39.5%, P<.05), whereas during PGE_1 infusion urinary NO_3^- excretion increased by only 32.3±17.2% (P=NS versus baseline; P<.05 between both treatments for area-under-the-curve analysis) (Fig 3A). Urinary cGMP excretion significantly increased (by 198.7±84.9%) after L-arginine infusion (P<.05) but only by 94.2±58.8% after PGE_1 (P=NS versus baseline). Area-under-the-curve analysis revealed no statistically significant difference in urinary cGMP excretion between both active infusions (Fig 3B). Placebo infusion induced no significant change in urinary NO_3^- or cGMP excretion rates (Fig 3).

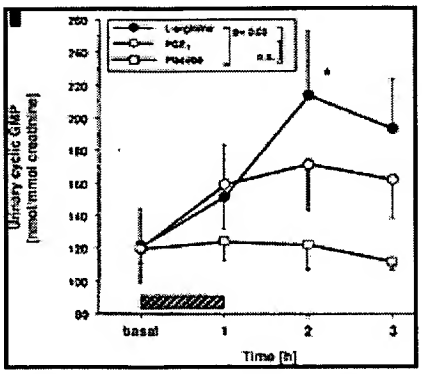

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Figure 3. Plots of urinary excretion rates of (A) nitrate and (B) cGMP in hourly intervals before (basal), during (first hour [1]), and after (second and third hours [2 and 3]) the infusion of L-arginine, PGE_1, or placebo. Values represent mean±SEM of 10 patients (L-arginine, PGE_1) or 6 patients (placebo). *P<.05 vs baseline in multiple ANOVA. Striped bar indicates the duration of the infusions.

No qualitative differences could be detected in the hemodynamic or biochemical responses to the infusions in subgroups of patients with or without diabetes, hypertension, or hypercholesterolemia.

Discussion

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The present study suggests that intravenous infusion of L-arginine, the precursor of endothelium-derived NO, induces peripheral vasodilation in the diseased limb of patients with severe peripheral arterial occlusive disease. This hemodynamic reaction is comparable to the one induced by a therapeutic dose of PGE_1, a prostaglandin used in the treatment of critical limb ischemia. Flow-induced vasodilation of conduit arteries is not involved in this hemodynamic effect, as femoral artery diameter remained constant during both L-arginine and PGE_1 infusion. However, infusion of L-arginine more strongly increased urinary nitrate and cGMP excretion rates, indicating that endogenous NO formation was enhanced during and after L-arginine infusion.

Evidence has been presented that the abnormal endothelium-dependent vasodilation in atherosclerosis is related to a reduced ability of the endothelium to produce and/or release biologically active vasorelaxant mediators NO[11 27 28] and prostacyclin.[19] Recently, we found that intravenous infusion of L-arginine induces vasorelaxation and inhibits platelet aggregation in healthy humans.[10] A hypotensive effect of L-arginine has also been shown by others in normal subjects[29] and in hypertensive and hypercholesterolemic patients.[17 30] These effects are suggested to be due to enhanced NO production, as assessed by quantification of NO_3^-, the major urinary metabolite of endogenous NO,[10 29 31] and of cGMP, the second messenger involved in NO-mediated effects.[10] Our present results suggest that in patients with severe peripheral arterial occlusive disease, intravenous L-arginine induces peripheral vasorelaxation in the diseased limb.

PGE_1, which binds to prostacyclin receptors and induces cAMP-dependent vasodilation, has been used in the pharmacotherapy of end-stage peripheral arterial occlusive disease.[21 22 32] Our present results showing a 31% increase in femoral artery blood flow confirm earlier findings by Hirai et al,[33] who found a ~38% increase in calf blood flow during intravenous PGE_1 infusion in patients with intermittent claudication.

Urinary nitrate excretion significantly increased during and after infusion of L-arginine and, less pronounced, of PGE_1. The urinary excretion rates of nitrate and cGMP have been found to be useful indicators of systemic NO production during physiological[24] or pharmacological[8 10 29] modulation of NO formation. Our present observation that urinary nitrate excretion was also enhanced by PGE_1, but not by placebo, may indicate that vasodilation may be a stimulator of NO production, probably via increased shear stress at the endothelial surface.[34] This suggests that the increased nitrate excretion may be a sum effect of NO synthase substrate provision (L-arginine) and increased shear stress (L-arginine and PGE_1).

Endothelium-dependent cholinergic relaxations in the forearm vascular bed of hypercholesterolemic patients have previously been shown to be improved by acute intravenous infusion of L-arginine,[17] but not by intra-arterial infusion.[18] In both studies, L-arginine had no effect on basal blood flow. In contrast to these studies in which local blood flow responses were monitored in the upper extremities, we investigated blood flow responses in the more severely diseased lower limb in patients with advanced atherosclerosis, and we observed a significant increase in blood flow with L-arginine. It is important to note that the defect in the endothelial L-arginine/NO/cGMP pathway appears to be reversible not only in early hypercholesterolemia[17] but also in advanced atherosclerosis, as shown here. Therefore, NO synthase substrate provision may be a new therapeutic approach to correct endothelial function in advanced peripheral arterial occlusive disease.

The pharmacological mechanism underlying the vasodilator effect of L-arginine remains unclear. Our present results strongly suggest that exogenously administered L-arginine stimulates NO formation. Others have shown that different mechanisms like vasodilator prostanoids,[35] histamine,[36] or insulin[17] are improbable to have contributed to this effect. In our present study, the hemodynamic effects of L-arginine were not different in the subgroups of patients with or without insulin-dependent diabetes mellitus, although this evidence is not conclusive, as we did not measure insulin secretion rates.

It is still unclear by which mechanism exogenous L-arginine increases NO formation, as intracellular L-arginine levels have been shown to be high enough (in the millimolar range) to saturate NO synthase, whose K_m has been determined in a cell-free enzyme preparation to be 2.9 μmol/L.[37] Based on this value, restitution of intracellular L-arginine levels alone seems improbable as a cause of increased NO production. However, it is not known whether the intracellular K_m is in the same order of magnitude in living tissues and whether other factors like cellular L-arginine uptake or intracellular compartimentalization affect L-arginine levels in the vicinity of the NO synthase. Moreover, the K_m may be different in cardiovascular disease. A recent report[38] suggested that an endogenous inhibitor of NO synthesis, N^G,N^G-dimethylarginine, is increased in serum from hypercholesterolemic rabbits. Dimethylarginine has previously been shown by Vallance and coworkers to be an endogenous inhibitor of NO synthase.[39] This inhibitory action might be competitively overcome by L-arginine, thereby stimulating NO production. However, data on plasma concentrations of dimethylarginines in hypercholesterolemic patients are not available.

In conclusion, results from the present study suggest that intravenous infusion of L-arginine induces peripheral vasodilation, which is probably mediated via NO, in patients with critical limb ischemia. Our study gives no conclusive evidence for any direct effect of L-arginine on the atherosclerotic process. Further studies will be needed to assess a potential therapeutic role of L-arginine in atherosclerotic disease and to elucidate its mechanism of action.

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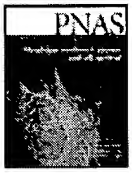

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Table 1. Characteristics of Patients Participating in the Study, Including Fontaine Stage Classification of Peripheral Arterial Disease and Risk Profile/Parallel Diseases

	L-Arginine/Prostaglandin E_1	Placebo
No. of patients	10	6
Age, y	68.3±3.1	62.6±3.8
Weight, kg	73.8±4.3	69.5±3.3
Height, cm	174.3±2.4	172.5±1.9
Fontaine stage III, n	2	2
Fontaine stage IV, n	8	4
Hypertension, n	3	1
Diabetes mellitus, n	5	3
Coronary heart disease, n	6	3
Carotid artery stenosis, n	6	1
History of smoking, n	5	5
Hypercholestolemia, n	6	4

Multiple entries are possible.

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Table 2. Plasma Cholesterol and Triglyceride Levels

	L-Arginine/Prostaglandin E_1	Placebo
Total cholesterol, mmol/L	5.7 ±0.4 (3.9-7.3)	5.5±0.8 (3.9-7.1)
LDL cholesterol, mmol/L	4.2±0.5 (2.2-6.0)	4.1±0.8 (2.4-5.8)
HDL cholesterol, mmol/L	1.3±0.1 (1.0-1.8)	1.1 ±0.1 (0.9-1.2)
Triglycerides, mmol/L	1.1 ±0.1 (0.5-1.8)	1.9±0.4 (1.0-2.9)

Values are given as mean±SEM (range) for 10 patients (L-arginine/prostaglandin E_1 group) or 6 patients (placebo group).

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Table 3. Comedications of Study Patients

	L-Arginine/Prostaglandin E_1	Placebo
Acetylsalicylic acid (100 mg/d)	10/10	6/6
Digoxin	2/10	1/6
Nitrates	3/10	2/6
Calcium antagonists	3/10	3/6
Diuretics	4/10	2/6
ACE inhibitors	1/10	...
Other	4/10	2/6

ACE indicates angiotensin-converting enzyme.

Values are given as number of patients taking the drug/number of patients in the treatment group (n=10 for L-arginine/prostaglandin E_1 group; n=6 for the placebo group).

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L-Arginine infusion decreases peripheral arterial resistance and inhibits platelet aggregation in healthy subjects

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1. L-Arginine is the physiological precursor of nitric oxide which induces vasodilatation and inhibits platelet aggregation by the formation of cyclic GMP.

2. In the present study we investigated the effects of an intravenous infusion of L-arginine (30 g, 30 min) compared with placebo on blood pressure, heart rate and peripheral haemodynamics in ten healthy male subjects. Cyclic GMP, NO_2^- and NO_3^- were determined in plasma and urine to assess NO production *in vivo* by a new, highly specific and sensitive gas chromatography–mass spectrometry method.

3. L-Arginine significantly decreased mean arterial blood pressure and increased heart rate. The effect was more pronounced on diastolic than on systolic blood pressure. This was due to a decreased peripheral arteriolar resistance, as in femoral artery Doppler sonography the arterial diameter was unchanged but blood flow was increased. These haemodynamic effects were not observed after placebo administration.

4. Urinary excretion of cyclic GMP increased by 65.4% after L-arginine and by 25.1% after placebo. Urinary NO_2^- excretion was near the threshold of detection. Urinary NO_3^- excretion increased by 79.7% after L-arginine. Plasma arginine levels increased nearly ten-fold after the L-arginine infusion, and plasma cyclic GMP increased by a similar rate as in urine. However, plasma NO_2^- and NO_3^- remained unchanged after both treatments, as did plasma α-atrial natriuretic peptide levels.

5. Platelet aggregation was inhibited by 32.7% after L-arginine ($P<0.05$), but was unchanged after placebo. Platelet intracellular cyclic GMP was increased by 43.0% after L-arginine, but not after placebo ($P<0.05$).

6. We conclude that intravenous L-arginine decreases peripheral arteriolar tone and inhibits platelet aggregation in healthy human subjects by enhancing nitric oxide formation and, concomitantly, cyclic GMP formation.

INTRODUCTION

Nitric oxide (NO) has been identified as the substance that accounts for the biological activity of endothelium-derived relaxing factor (EDRF) [1]. It is a potent vasodilator [2] and inhibitor of platelet aggregation and adhesion in animal and human tissues [3–5]. These actions are mediated by the intracellular second messenger cyclic GMP [6]. The amino acid L-arginine is the physiological precursor of NO, which is cleaved from one of the guanidino groups of L-arginine by the stereospecific enzyme NO synthase [7]. In aqueous solutions, NO is rapidly oxidized to nitrite (NO_2^-) and nitrate (NO_3^-); both NO_2^- and NO_3^- are present in plasma and are subsequently rapidly excreted into urine, mainly as NO_3^- [8]. NO has been shown to be the major source of urinary NO_2^- and NO_3^- in the absence of excess NO_3^- intake with food [9]. Consequently, the quantification of NO_2^- and NO_3^- has been suggested to be a suitable non-invasive method to determine EDRF activity *in vivo* [10, 11].

As inhibition of the L-arginine/NO pathway results in vasoconstriction [12, 13], a continuous basal release of endothelial NO has been postulated, which might be impaired in vascular diseases in which endothelial relaxation is diminished, i.e. hypercholesterolaemia [14], hypertension [15] and atherosclerosis [16, 17]. If the availability of L-arginine is considered as a rate-limiting factor for NO production *in vivo*, administration of L-arginine may be expected to result in increased NO formation. Indeed, a hypotensive action of L-arginine has been shown in normal subjects [18, 19] and in hypertensive patients by various groups [20, 21], whereas others did not find a hypotensive effect of L-arginine in normal subjects [22] or in hypertensive patients [23]. An NO synthase has also been shown to be present in human platelets [24], where NO is supposed to act as an endogenous inhibitor of platelet adhesion [3] and aggregation [5]. In this study we investigated the effects of L-arginine on systemic haemodynamics and on platelet aggregation *ex vivo* in healthy subjects and determined plasma and urinary concentrations of NO_2^-, NO_3^- and cyclic GMP as biochemical markers for NO formation *in vivo*.

Key words: L-arginine, cyclic GMP, endothelium, gas chromatography–mass spectrometry, haemodynamics, nitrate, nitric oxide, platelet aggregation.

Abbreviations: α-ANP, α-atrial natriuretic peptide; EDRF, endothelium-derived relaxing factor; PFB, pentafluorobenzyl.

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MATERIALS AND METHODS

Study design

Ten healthy male subjects (mean age 25.9 ± 1.0 years, height 183.3 ± 2.6 cm, weight 75.9 ± 2.7 kg) took part in this double-blind, randomized, placebo-controlled study. Each subject gave their written informed consent to participation in the study, which was approved by the Hannover Medical School Ethics Committee. The study consisted of 2 days: on 1 day L-arginine (Fresenius, Bad Homburg, Germany; 30 g dissolved in 300 ml of saline, pH 6.5) was infused into an antecubital vein over 30 min; on the other day placebo (300 ml of saline) was administered over the same time interval by an electrically driven motor pump (Perfusor; B. Braun, Melsungen, Germany). The two parts of the study were separated by a washout period of at least 7 days. Each study day was preceded by a 24 h interval during which the subjects were given a NO_3^--standardized diet (see below). At the beginning of each study day the subjects emptied their bladder. Mild oral volume loading (using demineralized water) was started with 3 ml/kg body weight initially and continued during the study period with 1–2 $ml\,h^{-1}\,kg^{-1}$ adjusted to the individual hourly urine volumes. Each participant remained in the supine position for 60 min before and 30 min after the infusion.

During 1 h before and 3 h after the beginning of the infusion, urine was collected at hourly intervals and immediately frozen for the determination of urinary NO_2^-, NO_3^- and cyclic GMP concentrations. Before and at the end of the infusion, a venous blood sample was drawn into EDTA for the determination of plasma cyclic GMP, α-atrial natriuretic peptide (α-ANP) and arginine levels, and another sample was drawn into 3.13% (w/v) sodium citrate for the determination of platelet aggregation.

Fifteen minutes before, during and for 10 min after the end of the infusion, blood pressure and heart rate were recorded every 5 min by the standard sphygmomanometric method using an automatic device (Boso digital II; Bosch and Sohn, Jungingen, Germany).

Blood flow in the femoral artery was determined by image-directed duplex ultrasonography before the infusion and at its end. Measurements were made with a DRF 400 image-directed duplex ultrasound system (Diasonics-Sonotron, Cologne, Germany) with a transducer combining 7.5 MHz B-mode imaging and 3 MHz pulsed Doppler beams. Blood flow volumes were automatically calculated as the vessel cross-sectional area multiplied by the time average volume from seven repeated measurements for each volume flow estimation.

NO_3^--standardized diet

For 24 h before each study day the participants were subjected to a standardized diet with a defined NO_3^- content. The diet consisted of 2000 ml/day of a fluid nutrient solution (Fresubin, Fresenius, Germany) containing 4184 kJ/l calorific value (13.8 g of carbohydrate, 3.8 g of protein and 3.4 g of lipid/100 ml) and 1.0 litres of mineral water. This diet contained 5.5 mg/l NO_3^- in the nutrient solution and 15.0 mg/l NO_3^- in the mineral water, which added up to a daily NO_3^- intake of 26.0 mg. The NO_2^- content of the diet was below 0.5 mg/l.

G.c.–m.s. measurement of NO_2^- and NO_3^-

Urinary NO_2^- and NO_3^- were determined as their pentafluorobenzyl (PFB) derivatives by g.c.–m.s. The derivatization procedure for the conversion of NO_2^- and NO_3^- to PFB-NO_2 was performed by a modification of the method described previously [25]. Briefly, 100 μl aliquots of urine were spiked with 250 ng of $^{15}NO_2^-$ as internal standard for NO_2^-, alkalinized with 10 μl of 4 mol/l NaOH, treated with 500 μl of cold acetone (−20°C) and centrifuged. PFB bromide (5 μl) was added to the decanted supernatant and the mixture was allowed to react for 75 min at 50°C. After cooling to room temperature, acetone was removed under nitrogen and the residue was extracted with 1 ml of toluene. The toluene phase was taken up and dried over Na_2SO_4. Aliquots (1 μl) thereof were then injected into the gas chromatograph–mass spectrometer.

For the determination of NO_3^-, 100 μl aliquots of urine samples were spiked with 250 ng of $^{15}NO_3^-$ as internal standard, acidified with 20 μl of 0.1 mol/l HCl and treated with 5 mg of cadmium for 10 min at room temperature. The suspension was then centrifuged, the supernatant decanted and derivatized as described above for NO_2^-.

G.c.–m.s. was performed on a triple-stage quadrupole mass spectrometer TSQ 45 interfaced with a gas chromatograph 9611 (Finnigan MAT, San Jose, CA, U.S.A.). An OV-1 fused silica capillary column (25 m × 0.25 mm inner diameter, 0.25 μm film thickness) from Machery-Nagel (Düren, Germany) was used with helium as the carrier gas (55 kPa). Negative ions were produced by chemical ionization using methane as the reactant gas (65 Pa) at an electron energy of 90 eV and an electron current of 0.2 mA. Quantification was performed by selected ion monitoring at *m/z* 46 endogenous NO_2^-/NO_3^- and *m/z* 47 for the internal standards. The detection limit of the method was 20 fmol of NO_2^- or NO_3^-. The intra-assay variability was below 3.8% [10].

Determination of cyclic GMP, creatinine, arginine and α-ANP

For the determination of cyclic GMP levels in urine or plasma, samples were thawed and centri-

fuged at 2500 *g* (4°C, 10 min). Supernatants were diluted 1:200 in PBS and acetylated by a mixture of acetic acid anhydride/triethylamine. The cyclic GMP content was measured by radioimmunoassay [26].

Urinary creatinine was determined spectrophotometrically with the alkaline picric acid method in an automatic analyser (Beckman, Galway, Republic of Ireland), and the urinary excretion of cyclic GMP, NO_2^- and NO_3^- was corrected by creatinine. Plasma arginine levels were determined spectrophotometrically after conversion to urea by the method of Bacchus and London [27]. For the determination of plasma α-ANP, plasma samples were centrifuged (2000 *g*, 43°C, 30 min) immediately after collection and stored in single-use aliquots at −25°C. After thawing, α-ANP was extracted by solid-phase extraction on C_8 columns (Amersham, Braunschweig, Germany). Plasma (1 ml) was acidified with 0.25 ml of 2 mol/l HCl and centrifuged before loading on to the columns. The columns were washed with water/trifluoroacetic acid (0.1%, v/v), and α-ANP was eluted with 60% acetonitrile/water containing 0.1% (v/v) trifluoroacetic acid. The eluent was evaporated to dryness with nitrogen and reconstituted in 250 μl of assay buffer [0.025 mol/l phosphate (pH 7.2) containing 0.1% (w/v) sodium azide]. Aliquots (100 μl) were taken for analysis in duplicate using a commercially available ^{125}I-α-ANP radioimmunoassay kit (Amersham). The sensitivity of the assay, defined as the amount of human α-ANP needed to reduce zero dose binding by two SDs, was less than 1 fmol/tube (4 fmol/ml).

Platelet aggregation

Platelet-rich plasma was generated by centrifugation at 200 *g* for 10 min. Platelet-poor plasma was prepared from the remaining volume of blood by centrifugation at 2000 *g* for 10 min. Platelet aggregation was monitored at 37°C by the method of Born and Cross [28] using an Apact dual-channel aggregometer (LAbor, Hamburg, Germany) as described previously [29]. The aggregometer was adjusted before each test so that in each subject the value for light transmission for platelet-rich plasma was 0% and that for platelet-poor plasma was 100%. Aggregation was induced in duplicate using a final concentration of 2 μmol/l ADP, and was monitored for 3 min. Thereafter, the reaction was quenched by shock-freezing the platelet-rich plasma in liquid nitrogen. The samples were stored at −20°C until analysis of cyclic GMP by specific radioimmunoassay.

Drugs

L-Arginine hydrochloride and the fluid nutrient solution (Fresubin) were kindly supplied by Fresenius AG (Bad Homburg, Germany). The reagent kits for the determination of arginine levels, urea standard, L-arginine standard, trichloroacetic acid and ADP were purchased from Sigma (Munich, Germany).

Calculations and statistics

The results are expressed as means ± SEM. Statistical analysis was performed by using Student's *t*-test for paired or unpaired observations as appropriate. Differences of biochemical parameters before, during and after the infusions were analysed for statistical significance using one-way analysis of variance followed by the Fisher least significant difference test for the comparison of different means. A P value of <0.05 was considered to be significant in all cases.

RESULTS

Haemodynamic effects of L-arginine

During the infusion of L-arginine, blood pressure progressively decreased and heart rate increased (Fig. 1). The effect was more pronounced on diastolic (−6.7 mmHg; $P<0.001$) than on systolic blood pressure (−4.3 mmHg; $P<0.05$). After the end of the infusion, blood pressure and heart rate returned to baseline values within 5 min. The area under the diastolic blood pressure curve was significantly smaller and the area under the heart rate curve was significantly larger for the L-arginine treatment than for placebo ($P<0.05$), whereas for systolic blood pressure the difference between the treatments was not significant ($P=0.710$).

Blood flow in the femoral artery was significantly enhanced by L-arginine (43.5%; $P<0.02$), whereas placebo induced no change in arterial blood flow (Fig. 2). The increase in blood flow induced by L-arginine was due to an increased flow velocity (6.5 ± 0.7 to 9.3 ± 1.0 cm/s; $P<0.002$), whereas the femoral artery diameter was unchanged by the treatment.

Urinary NO_2^-, NO_3^- and cyclic GMP excretion

Hourly urine volume was increased both by L-arginine and placebo, but creatinine clearance showed only a minor, insignificant increase after L-arginine (116.2 ± 16.9 to 135.3 ± 13.1 ml/min) and remained constant after placebo.

Basal urinary excretion of NO_3^- was 53.1 ± 6.0 μmol/mmol of creatinine. NO_2^- excretion was near the detection limit before and after the infusions. Therefore, urinary excretion rates are expressed as NO_3^-. Urinary excretion of both NO_3^- and cyclic GMP significantly increased 1.6–1.8-fold over baseline during and after infusion of L-arginine ($P<0.05$) (Figs. 3 and 4). During and after placebo, urinary NO_3^- remained unchanged, whereas cyclic GMP excretion increased about 1.3-fold over base-

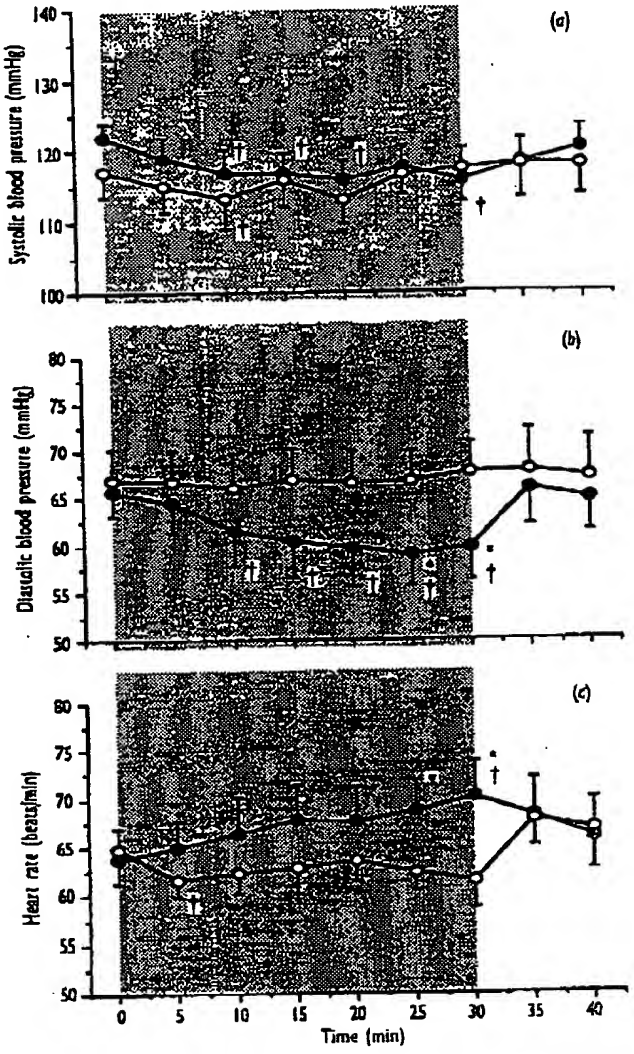

Fig 1. Effects of L-arginine (●, 30 g, 300 ml) and placebo (○, 300 ml of saline) infused for 30 min in ten healthy male subjects, on (a) systolic blood pressure, (b) diastolic blood pressure and (c) heart rate. Values are shown as means ± SEM. Statistical significance: *$P<0.05$ for L-arginine compared with placebo; †$P<0.05$ compared with before infusion. The shaded area indicates the duration of the infusions.

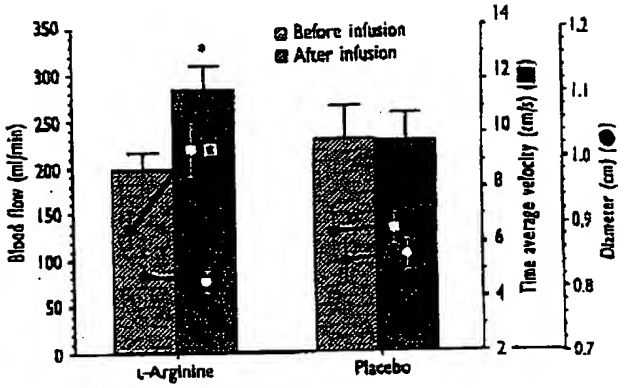

Fig. 2. Changes in blood flow in the femoral artery before and immediately after the end of infusion of 30 g of L-arginine or placebo in ten healthy male subjects. Blood flow was determined from vessel diameter (●) and time average velocity (■) using Duplex sonography. Values are given as means ± SEM. Statistical significance: *$P<0.05$ compared with before infusion.

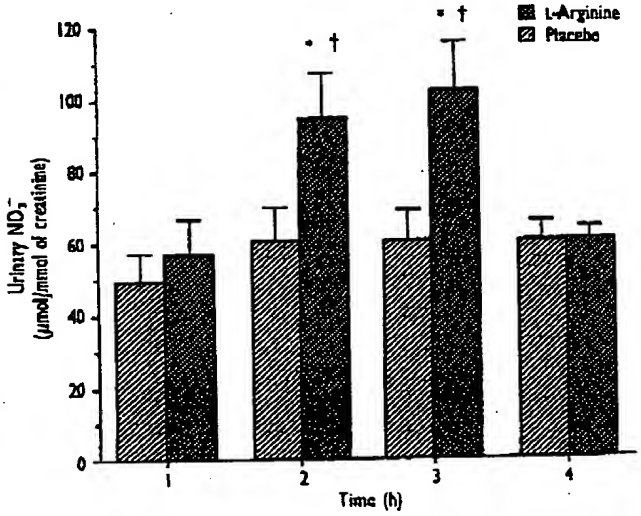

Fig. 3. Changes in the urinary excretion of NO_3^- before (1st hour), during (2nd hour) and after (3rd and 4th hour) the infusion of 30 g of L-arginine or placebo in ten healthy subjects. Measurements were corrected for urinary creatinine and are given as means ± SEM. Statistical significance: *$P<0.05$ for L-arginine compared with placebo; †$P<0.05$ compared with before infusion.

line ($P<0.05$). The increase in urinary NO_3^- excretion correlated with the decrease in diastolic blood pressure ($r=0.496$, $P<0.05$) (Fig. 5*a*). Urinary excretion of NO_3^- and cyclic GMP showed a linear correlation ($r=0.327$, $P<0.05$) (Fig. 5*b*).

Platelet aggregation and platelet cyclic GMP content

Maximal platelet aggregation induced by ADP was inhibited by 32.7% after L-arginine ($P<0.05$) (Fig. 6*a*). Platelet cyclic GMP content increased by 43.0% after L-arginine ($P<0.05$) (Fig. 6*b*). After the infusion of placebo, both platelet cyclic GMP content and maximal aggregation were not significantly changed.

Effects on plasma cyclic GMP, α-ANP and arginine levels

The influence of both treatments on plasma parameters is given in Table 1. Infusion of L-arginine induced a tenfold increase in plasma arginine concentration ($P<0.001$), whereas during the infusion of saline no significant variation in plasma arginine concentrations was observed. Plasma cyclic GMP increased by 53.0% after L-arginine and by 24.3% after placebo ($P<0.01$). Neither the L-arginine infu-

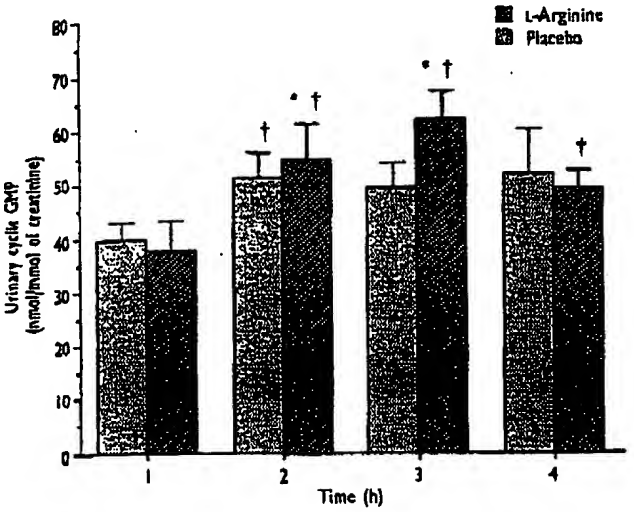

Fig. 4. Changes in the urinary excretion of cyclic GMP before (1st hour), during (2nd hour) and after (3rd and 4th hour) the infusion of 30 g of L-arginine or placebo in ten healthy subjects. Measurements were corrected for urinary creatinine and are given as means ± SEM. Statistical significance: *$P < 0.05$ for L-arginine compared with placebo; †$P < 0.05$ compared with before infusion.

sion nor placebo influenced plasma α-ANP levels, which were well within the physiological range in all subjects.

DISCUSSION

The present study shows that the intravenous administration of L-arginine in healthy subjects decreases blood pressure, raises heart rate and inhibits platelet aggregation. These effects were accompanied by an increase in urinary NO_3^- and cyclic GMP excretion. Kanno et al. [19] have previously shown that intravenous L-arginine decreased mean arterial blood pressure in humans. By contrast, we differentiate between diastolic and systolic blood pressure and show that diastolic blood pressure is more strongly decreased than systolic blood pressure. This effect was most likely to be due to a reduced peripheral arterial resistance, as indicated by our results of duplex sonography. These results are in agreement with findings from animal studies showing that endothelial NO formation from L-arginine plays a fundamental role in the regulation of vascular resistance [12, 30, 31].

Intravenous L-arginine also inhibited platelet aggregation via an increase in intraplatelet cyclic GMP, showing that its biological effects are similar to those of endogenous EDRF/NO [1, 32, 33]. Urinary NO_3^- and cyclic GMP excretion was enhanced after L-arginine, but this effect showed some delay compared with the biological effects: for both parameters the peak excretion was reached after the end of the infusion. This effect, which was also noted by Kanno et al. [19], may be explained by a delay between the endothelial production of NO,

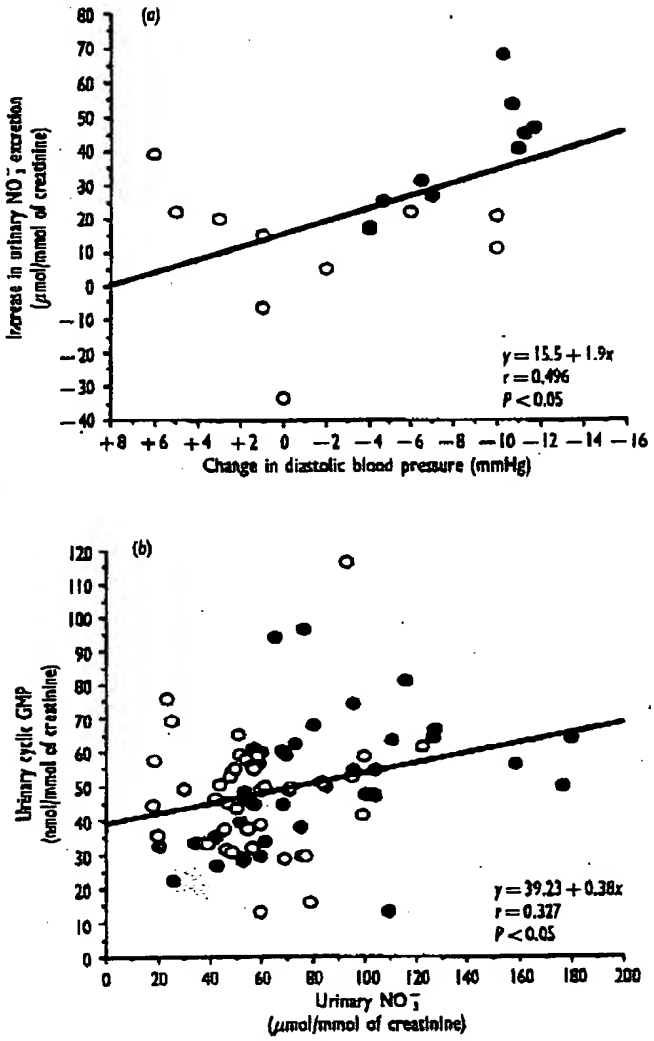

Fig. 5. Correlation between the increase in urinary NO_3^- excretion and the decrease in diastolic blood pressure (a) and the increase in urinary cyclic GMP excretion (b) during the infusion of L-arginine (●) or placebo (○) in ten healthy subjects.

the onset of its biological actions in nearby cells, and the excretion of its inactivation product and second messenger, respectively, by the kidneys.

A basal release of endothelium-derived NO has been demonstrated in the past to be involved in the physiological regulation of blood pressure, mainly in studies showing that the blockade of NO synthesis increases blood pressure [12, 13]. However, there has been a long-standing controversy as to whether NO synthase is saturated by endogenous L-arginine: supplementation of L-arginine in animals has yielded conflicting results, depending upon the species and vascular preparation used [12, 34]. Some studies in humans also revealed that L-arginine reduced blood pressure [18, 19, 35], but others reported a lack of hypotensive effects of L-arginine in humans [22, 23]. Furthermore, Calver et al. [36] reported that D-arginine also induced an

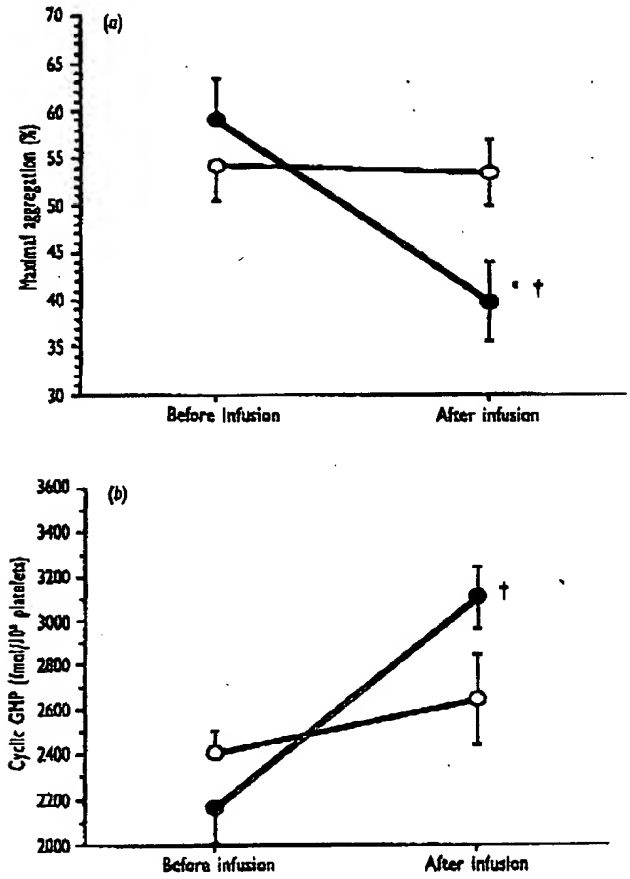

Fig. 6. Platelet aggregation induced by 2 μmol/l ADP (*a*) and platelet cyclic GMP content (*b*) before and after the infusion of L-arginine (●) and placebo (○). Values are means ± SEM for ten subjects. Statistical significance: *$P < 0.05$ for L-arginine compared with placebo; †$P < 0.05$ compared with before infusion.

Table 1. Plasma concentrations of arginine, cyclic GMP and α-ANP before and after infusion of L-arginine and placebo in ten healthy subjects. L-Arginine (30 g, 300 ml) or placebo (300 ml of saline) were infused into ten healthy male subjects during 30 min. Blood was drawn immediately before and after the infusions. Values are means ± SEM. Statistical significance: *$P < 0.05$ compared with placebo, †$P < 0.05$ compared with before infusion.

	L-Arginine		Placebo	
	Before	After	Before	After
Arginine (μmol/l)	152.1 ± 21.8	1378.1 ± 129.0*†	125.4 ± 37.9	111.3 ± 31.4
Cyclic GMP (pmol/l)	2628.4 ± 155.5	4326.5 ± 404.8*†	2876.8 ± 207.2	3574.9 ± 340.1†
α-ANP (pmol/l)	7.18 ± 0.39	7.45 ± 0.45	6.81 ± 0.27	7.07 ± 0.29

unspecific increase in forearm blood flow when administered intra-arterially in sufficiently high doses. However, the concentrations they reached were up to 10-fold higher than the arginine plasma concentrations in the present study.

Other, alternative, explanations for the vasodilatory effects of L-arginine have been largely excluded by other investigators. The pH of L-arginine hydrochloride solutions has been shown by Calver et al. [36] not to influence the pH of venous blood, making an acidity-induced vasodilatation improbable. Arginine may induce the release of histamine [20], but the addition of an H_1-receptor antagonist did not attenuate the vasodilator actions of arginine [21]. However, an implication of other factors, such as insulin or glucagon, which are released by L-arginine [37], cannot be excluded at present, although our finding that L-arginine increased platelet cyclic GMP content and inhibited platelet aggregation *ex vivo* strongly supports the idea that biologically active NO is produced from exogenous L-arginine *in vivo*.

EDRF or NO has previously been shown by ourselves and others [33, 34, 38, 39] to inhibit platelet adhesion and aggregation *in vitro*. An NO synthase has also been shown to be present in human platelets, where it is suggested to act as an intrinsic inhibitor of platelet activation [24]. EDRF has also been shown to inhibit platelet aggregation *in vivo* in an animal model [40], but intra-arterial infusion of acetylcholine in humans did not affect whole blood aggregation *ex vivo* [41]. By contrast, our present data show for the first time that aggregation of platelet-rich plasma *ex vivo* is significantly inhibited by 32.7% after intravenous L-arginine. This effect, although much weaker than the anti-aggregatory effects of acetylsalicylic acid [29] or prostacyclin [42], may contribute to the known anti-atherosclerotic effect of L-arginine [43].

A direct determination of endothelium-derived NO in man is currently not possible, owing to its short half-life caused by oxidation to NO_2^- and NO_3^- in aqueous media. Determination of the stable oxidation products NO_2^- and NO_3^- was proposed by ourselves and others [10, 11] to assess NO production *in vivo*. Our newly developed g.c.–m.s. method is characterized by a high degree of accuracy and sensitivity. Its reproducibility was found to be greater than 96%, and possible interferences by other inorganic anions are excluded. With the help of this method, we could demonstrate a 79.7% increase in urinary NO_3^- after the intravenous administration of L-arginine, whereas after placebo urinary NO_3^- excretion was not significantly changed. We had previously shown that the urinary excretion rates of NO_3^- and cyclic GMP correlated with the blood pressure effects in rats treated with L-arginine and with the inhibitor of NO formation, N^G-nitro-L-arginine methyl ester [44].

However, to ensure that urinary NO_3^- reliably mirrors NO formation by the constitutive isoenzyme of NO synthase, the dietary intake of NO_3^- should be standardized, if not reduced, because the NO_3^- content of food varies widely and is a major factor of variability of urinary NO_3^- excretion rates [45]. It is important to stress that in our study

dietary NO_3^- intake was standardized by giving the subjects a defined diet for 24 h before each study day. Urinary clearance of ingested NO_3^- was reported to be essentially completed within this time span [46].

Plasma cyclic GMP was about twofold higher after L-arginine than after placebo infusion, indicating that about half the increase in cyclic GMP after the infusion of L-arginine may have been induced via an L-arginine-specific mechanism, i.e. NO formation. The same amount of cyclic GMP increase could be ascribed to NO synthesis in the isolated perfused rat kidney after stimulation with acetylcholine [47]. Stimulation of NO formation *in vivo* also induced a rise in urinary cyclic GMP excretion in the anaesthesized rat [31]. However, cyclic GMP is no specific parameter for NO synthase activity, as other humoral compounds, such as α-ANP, also increase its formation by activating the particulate isoenzyme of guanylate cyclase [48], e.g. during acute plasma volume expansion [49]. Therefore, the increase in cyclic GMP that occurred after placebo might have been due to increased α-ANP formation. However, in our study we could not detect an increase in plasma α-ANP.

In conclusion, intravenous administration of L-arginine increases NO and, concomitantly, cyclic GMP production in healthy human subjects with a defined diet. L-Arginine reduces blood pressure due to a decreased peripheral vascular resistance, and inhibits platelet aggregation *ex vivo* in normal human subjects. The determination of urinary NO_3^- by g.c.–m.s. is a sensitive and specific method for the non-invasive determination of NO formation *in vivo* in humans.

ACKNOWLEDGMENTS

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Inhaled Nitric Oxide Inhibits Human Platelet Aggregation, P-Selectin Expression, and Fibrinogen Binding In Vitro and In Vivo

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Background—Recent data suggest that inhaled NO can inhibit platelet aggregation. This study investigates whether inhaled NO affects the expression level and avidity of platelet membrane receptors that mediate platelet adhesion and aggregation.

Methods and Results—In 30 healthy volunteers, platelet-rich plasma was incubated with an air/5% CO_2 mixture containing 0, 100, 450, and 884 ppm inhaled NO. ADP- and collagen-induced platelet aggregation, the membrane expression of P-selectin, and the binding of fibrinogen to the platelet glycoprotein (GP) IIb/IIIa receptor were determined before (t_0) and during the 240 minutes of incubation. In addition, eight patients suffering from severe adult respiratory distress syndrome (ARDS) were investigated before and 120 minutes after the beginning of administration of 10 ppm inhaled NO. In vitro, NO led to a dose-dependent inhibition of both ADP-induced (3±3% at 884 ppm versus 70±6% at 0 ppm after 240 minutes; $P<.001$) and collagen-induced (13±5% versus 62±5%; $P<.01$) platelet aggregation. Furthermore, P-selectin expression (36±7% of t_0 value; $P<.01$) and fibrinogen binding (33±11%; $P<.01$) were inhibited. In patients with ARDS, after two who did not respond to NO inhalation with an improvement in oxygenation had been excluded, an increase in plasma cGMP, prolongation of in vitro bleeding time, and inhibition of platelet aggregation and P-selectin expression were observed, and fibrinogen binding was also inhibited (19±7% versus 30±8%; $P<.05$).

Conclusions—NO-dependent inhibition of platelet aggregation may be caused by a decrease in fibrinogen binding to the platelet GP IIb/IIIa receptor. **(*Circulation*. 1998;97:1481-1487.)**

Key Words: platelets ■ P-selectin ■ fibrinogen ■ nitric oxide ■ respiration

Nitric oxide has been found to relax vascular smooth muscle and to inhibit platelet adhesion and platelet aggregation in vitro.[1-3] Inhaled NO decreases elevated pulmonary arterial pressure and pulmonary vascular resistance and improves oxygenation. This principle has been used successfully to treat ARDS, persistent pulmonary hypertension of the newborn, and pulmonary hypertension in patients who have undergone cardiac surgery.[4-6] Despite rapid inactivation of inhaled NO by hemoglobin and high selectivity for the pulmonary vascular system,[5,7] recent data suggest that inhaled NO also has systemic effects on hemostasis by inhibiting platelet function. During NO inhalation, bleeding time was prolonged in animals and in healthy volunteers, and platelet aggregation was inhibited in patients with ARDS.[8-10] In addition, inhibition of platelet aggregation was also observed during NO inhalation after acute massive pulmonary embolism in pigs.[11]

Although several NO-releasing compounds have been investigated and the platelet inhibitory effect has been shown to be dose dependent in vitro and in vivo,[12-16] it is currently unknown whether the inhibitory effect of inhaled NO on platelet function is also dose related. Furthermore, the mechanisms by which inhaled NO may affect platelet function are not completely understood. Inhibition of fibrinogen binding to the platelet membrane via an increase in intracellular cGMP concentration has been postulated to be responsible for the inhibition of platelet adhesion and aggregation.[2,3,17] However, fibrinogen binding to human platelets during NO inhalation has not been investigated in detail. The present study was performed to investigate the dose dependency of platelet inhibition in vitro and in patients with ARDS by use of platelet aggregation studies, determination of in vitro bleeding time, and flow cytometry to elucidate the mechanism by which inhaled NO affects platelet function.

Methods

NO Gas In Vitro

Platelet Preparation and NO Administration

After approval from the ethics committee and informed consent from the study subjects had been obtained, venous blood was drawn from

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Selected Abbreviations and Acronyms

ARDS = acute respiratory distress syndrome
FC = final concentration
GP = glycoprotein
PRP = platelet-rich plasma

an antecubital vein in 30 healthy volunteers (26±5 years old) who had taken no drugs that could affect platelet function during the previous 14 days. After the first 10 mL had been discarded, samples were carefully drawn into plastic tubes containing 3.8% sodium citrate (Monovette, Sarstedt). Samples were centrifuged immediately at 150*g* for 10 minutes to obtain PRP and subsequently at 2500*g* for 10 minutes to obtain platelet-poor plasma. The platelet concentration was standardized to 300/nL by addition of platelet-poor plasma to PRP. Aliquots of 10 mL PRP were put into a tube consisting of a dialysis membrane (Spectra/Por, Spectrum) with a molecular weight cutoff at 1000 daltons. A central venous catheter (Certo 358, Braun) was placed into the tube to allow PRP sampling during the study period. The tube was fixed in a larger glass container filled with Tyrode's solution. Temperature was maintained at 37°C in a water bath. An air/oxygen mixture (Fio_2=0.3) with 5% CO_2 was administered to the buffer at a flow rate of 1.5 L/min. The indirect method of administering gas to PRP was chosen to prevent mechanical platelet activation by gas bubbles (Fig 1).

Ten minutes after gas administration was begun, 0, 100, 450, and 884 ppm NO were added for 240 minutes. NO was supplied by Messer Griesheim at an original gas concentration of 884 ppm NO in N_2. Gas samples for measurement of the concentrations of NO and NO_2 were taken continuously at the inlet and outlet tubes and analyzed by chemiluminescence ($NO/NO_2/NO_x$ analyzer CLD 700 AL, Zellweger-Ecco).

Determination of Platelet Aggregation

To determine platelet aggregation, PRP samples were carefully withdrawn from the central venous catheter at the beginning (t_0) and after 20 (t_{20}), 40 (t_{40}), and every 60 minutes of NO administration (t_{60}, t_{120}, t_{180}, and t_{240}) for an overall time of 240 minutes. ADP-induced (Mölab; FC, 5 μmol/L) and collagen-induced (Mölab; FC, 0.19 mg/mL) platelet aggregation were measured in a four-channel platelet amplifier (PAP-4, Biodata Corp) at 37°C according to the method described by Born.[18] Each test was carried out in duplicate, and the mean value of both measurements was recorded. Aggregation was quantified by measurement of the maximal extent of light transmission (maximal aggregation, measured as percentage).

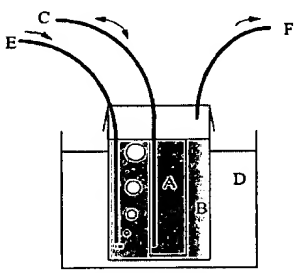

Figure 1. Experimental setting. Tube containing PRP (A) consists of a dialysis membrane with a molecular weight cutoff at 1000 daltons. It is fixed in a larger, gas-light container (B) filled with Tyrode's solution. A central venous catheter (C) was used to take PRP samples during study. Temperature was maintained with a warming water bath (D). Air/oxygen/carbon dioxide/NO mixture was administered to buffer via inlet tube (E). Gas samples from E and from outlet tube (F) were taken to determine NO concentrations.

Determination of P-Selectin Expression and Fibrinogen Binding

To determine P-selectin expression and fibrinogen binding to the platelet GP IIb/IIIa receptor, additional PRP samples were carefully taken from the tube containing PRP at t_0, t_{60}, t_{120}, t_{180}, and t_{240} during administration of 0, 100, and 884 ppm NO. There were no centrifugation or vortexing steps, so that in vitro platelet activation was avoided. To determine P-selectin expression, PRP was diluted in HEPES solution, and to measure fibrinogen binding, in Tyrode's buffer (Roth). P-selectin and fibrinogen were measured after stimulation with ADP (Mölab, FC, 2 μmol/L). The samples were incubated with the specific antibodies CD62P CloneAC1.2 (Becton Dickinson) to determine P-selectin expression and anti-human fibrinogen FITC-conjugated chicken antibody (Biopool) to determine fibrinogen binding for 20 minutes in the dark at 22°C. After fixation with Cellfix (Becton Dickinson), the samples were measured in a flow cytometer within 6 hours. The platelet population was analyzed at a low flow rate and identified on the basis of forward and sideways scatter characteristics. For each sample, 10 000 platelets were collected. Data were analyzed with LYSIS II software (FACScan, Becton Dickinson).[19-21]

NO Gas in Older Volunteers

Because of the age imbalance between the normal volunteers in the in vitro study and the ARDS patients, PRP from five additional volunteers (55 to 65 years old) was incubated with 0, 100, and 884 ppm NO. Platelet aggregation, P-selectin expression, and fibrinogen binding were investigated as described above.

50 ppm NO In Vitro

The NO concentrations used in the in vitro investigation were relatively high (100 to 884 ppm). To investigate a lower NO concentration, PRP from five additional volunteers was incubated with 50 ppm for 240 minutes. Platelet aggregation, P-selectin expression, and fibrinogen binding were investigated at t_{180} and t_{240} as described above.

Inhaled NO in Patients With ARDS

Patients and NO Administration

After approval by the ethics committee and informed consent from the relatives had been obtained, eight surgical intensive care patients (64±4 years old; seven men and one woman) diagnosed with ARDS who fulfilled the criteria of ARDS according to the consensus conference[22] were investigated. Two patients had been admitted with multiple trauma, four had undergone coronary revascularization, major vascular surgery had been performed in one, and one had developed ARDS after major abdominal surgery. Exclusion criteria were suspected or confirmed intracranial hemorrhage, leukopenia, and a previous history of severe chronic kidney, liver, or lung disease.

All patients were sedated with intravenous midazolam (Dormicum; Hoffmann-LaRoche) and fentanyl (Janssen) and mechanically ventilated to ensure an arterial carbon dioxide tension ($PaCO_2$) between 40 and 50 mm Hg (EVITA 2, Dräger). In all patients, hemodynamic measurements were performed with a pulmonary artery catheter (93-631-5.5F, Baxter Healthcare Corp) and a radial arterial catheter (20 gauge; Abbocath-T, Abbott), and data were continuously displayed on a multichannel oscillograph (Vicom SMU-612, Hellige). After the patients had been selected, 10 ppm inhaled NO (Messer Griesheim) were administered with a commercially supplied administration unit connected to the ventilator (NOdomo, Dräger). Inspiratory and expiratory concentrations of NO and NO_2 were analyzed continuously at the proximal end of the endotracheal tube with the $NO/NO_2/NO_x$ analyzer (Zellweger-Ecco).

To analyze platelet function, the first 10 mL of arterial blood was discarded, and thereafter samples were carefully withdrawn into plastic tubes (Monovette) before (t_0) and 120 minutes after (t_{120}) NO administration was initiated.

Determination of In Vitro Bleeding Time

With a platelet function analyzer (PFA 100, Dade), the in vitro bleeding time was determined according to the following method, described by Kratzer and Born[23]: At a constant negative pressure, samples of citrated whole blood (800 μL) are suctioned through a small capillary and a filter membrane with a diameter of 150 μm. The filter membrane is covered with collagen and soaked with epinephrine. During movement through the capillary, platelets adhere and aggregate at the filter membrane, diminishing blood flow until it stops. The total time in seconds of the blood flow is called in vitro bleeding time and is measured electronically.

Determination of Platelet Aggregation

As described above, whole blood (8 mL) was centrifuged at 500*g* for 5 minutes and subsequently at 2500*g* for 10 minutes to prepare PRP, and ADP-induced platelet aggregation (FC, 5 μmol/L) was measured. Because of the short half-life of NO and its rapid inactivation after contact with hemoglobin, all samples were centrifuged immediately and measured within 30 minutes after sampling.

Determination of P-Selectin Expression and Fibrinogen Binding

To determine P-selectin expression and fibrinogen binding to the platelet membrane in patients during NO inhalation, whole-blood samples (20 μL) were measured as described above. In these patients, P-selectin expression was determined without (basal P-selectin) and after activation with ADP (FC, 2 μmol/L).

Measurement of Platelet Count, White Blood Cell Count, and Plasma cGMP

Platelet count and white blood cell count were measured with a Coulter Counter STKS (Coulter Electronics). Arterial plasma samples that had been stored deep frozen (−80°C) until measurement were used to determine plasma cGMP levels with a commercially supplied enzyme immunoassay used according to the manufacturer's guidelines (Immunodiagnostik).

Statistical Analysis

All results are given as mean±SEM. In addition, the values are also given as a percentage of the baseline value (t_0, given as 100%) to demonstrate the relative changes during the time course of P-selectin expression and fibrinogen binding. Statistical analysis was performed with one-way ANOVA for repeated measurements, followed by the Scheffé test to demonstrate changes in platelet aggregation, P-selectin expression, and fibrinogen binding and to analyze the differences between the two groups. For additional data analysis, the Wilcoxon signed rank test for paired samples was used in the ARDS patients. To analyze whether the effect of NO on platelet function was due to changes in fibrinogen binding, the changes in aggregation (aggregation ratio) and in fibrinogen binding (fibrinogen-binding ratio) during incubation with 884 ppm NO were correlated by the Pearson test. A value of $P<.05$ was considered to be statistically significant.

Results

NO Gas In Vitro

In the control group, ADP-induced platelet aggregation did not change during the study (72±5% at t_0, 73±7% at t_{20}, 67±7% at t_{40}, 68±6% at t_{60}, 65±5% at t_{120}, 67±7% at t_{180}, and 70±6% at t_{240}). During administration of 100 ppm NO, ADP-induced platelet aggregation was inhibited, reaching statistical significance at time points t_{120} (46±7%, $P<.05$), t_{180} (41±7%, $P<.05$), and t_{240} (26±4%, $P<.01$). Administration of 450 ppm NO significantly inhibited ADP-induced platelet aggregation at t_{60} (43±10%, $P<.05$), t_{120} (32±7%, $P<.01$), t_{180} (23±7%, $P<.01$), and t_{240} (12±4%, $P<.001$). The highest NO concentration (884 ppm) resulted in the most pronounced inhibition of platelet aggregation: 37±5% at t_{20} ($P<.01$), 38±6% at t_{40} ($P<.01$), 29±5% at t_{60} ($P<.001$), 15±5% at t_{120} ($P<.001$), 9±5% at t_{180} ($P<.001$), and 3±3% at t_{240} ($P<.001$) (Fig 2). In accordance with these findings, collagen-induced platelet aggregation also was dose-dependently inhibited during NO administration, reaching statistical significance during administration of 450 and 884 ppm NO. During administration of 450 ppm (884 ppm) at t_{120}, t_{180}, and t_{240}, collagen-induced platelet aggregation was 49±8% (55±3%, $P<.05$), 45±6% (37±3%, $P<.01$), and 25±10% (13±5%, $P<.001$), respectively (Fig 3).

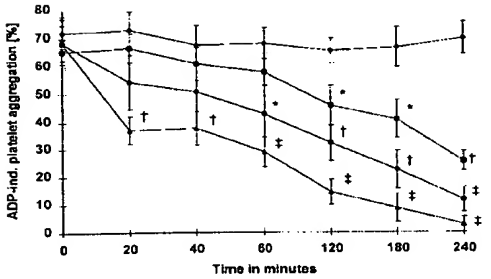

Figure 2. ADP-induced platelet aggregation (FC, 5 μmol/L) at 0, 20, 40, 60, 120, 180, and 240 minutes. NO was administered in vitro at 0 (diamonds; control), 100 (squares), 450 (circles), and 884 (triangles) ppm (mean±SEM; *$P<.05$, †$P<.01$, ‡$P<.001$ vs control).

In vitro administration of 100 ppm NO led to a significant inhibition of P-selectin expression at t_{240} (76±12% versus t_0 value, $P<.05$). During incubation with 884 ppm NO, P-selectin expression was inhibited at t_{60}, t_{120}, t_{180}, and t_{240} (77±12%, $P<.05$; 73±13%, $P<.05$; 67±23%, $P<.01$; and 36±7%, $P<.01$, respectively) (Fig 4). Furthermore, in accordance with the other findings, NO administration to PRP resulted in a significant inhibition of platelet fibrinogen binding to the GP IIb/IIIa receptor. Compared with t_0 values, during administration of 450 ppm (884 ppm) NO, fibrinogen binding was 73±17% at t_{120} (66±16%, $P<.05$), 57±30% at t_{180} (51±15%, $P<.01$), and 48±28% at t_{240} (33±11%, $P<.01$) (Fig 5). Moreover, a significant correlation between the aggregation ratio and the fibrinogen-binding ratio was found during NO incubation (Fig 6).

NO-induced dose-related inhibition of platelet aggregation, P-selectin expression, and fibrinogen binding was also observed in the older study population (Table 1). In comparison

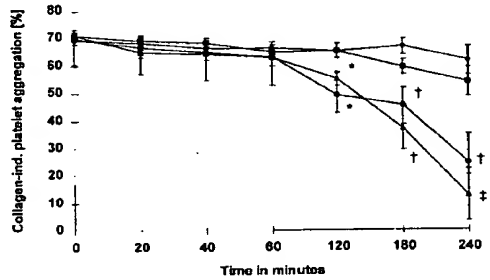

Figure 3. Collagen-induced platelet aggregation (FC, 0.19 mg/dL) at 0, 20, 40, 60, 120, 180, and 240 minutes. NO was administered in vitro at 0 (diamonds; control), 100 (squares), 450 (circles), and 884 (triangles) ppm in vitro (mean±SEM; *$P<.05$, †$P<.01$, ‡$P<.001$ vs control).

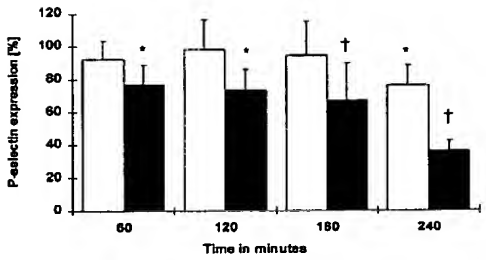

Figure 4. P-selectin expression after activation with ADP (FC, 2 μmol/L) as a percentage of baseline value at 60, 120, 180, and 240 minutes. NO was administered in vitro at 100 (open bars) and 884 (solid bars) ppm (mean±SEM; *$P<.05$, †$P<.01$ vs control).

with the younger population, no statistically significant differences were observed.

Incubation with 50 ppm NO also led to a significant inhibition of ADP-induced platelet aggregation in vitro. In addition, a slight inhibition in P-selectin expression and a significant inhibition in fibrinogen binding were observed (Table 2).

Inhaled NO in Patients With ARDS

Two patients suffering from ARDS who had undergone cardiac surgery did not respond to NO inhalation with an improvement in oxygenation. NO was withdrawn in these patients, and they were excluded from the study. In the remaining six patients, the in vitro bleeding time was prolonged (99±13 seconds at t_{120} versus 71±11 seconds at t_0, $P<.05$) and ADP-induced platelet aggregation was inhibited (19±3% versus 26±2%, $P<.05$) during inhalation of 10 ppm NO. In addition, NO inhalation significantly inhibited both basal and ADP-stimulated P-selectin expression: 6.7±1.7% versus 10.1±2.2% ($P<.05$) and 43±6% versus 57±7% ($P<.05$), respectively. In accordance with these findings, the fibrinogen binding to the platelet GP IIb/IIIa receptor was also inhibited during NO inhalation in patients with ARDS (19±7% versus 30±8%, $P<.05$). In addition, NO therapy led to a significant increase in plasma cGMP levels (8.1±1.6 versus 4.4±1.2 ng/mL, $P<.05$) but had no influence on platelet and white blood cell count (Table 3).

Discussion

The present study demonstrates that inhaled NO inhibits platelet aggregation, P-selectin expression, and fibrinogen

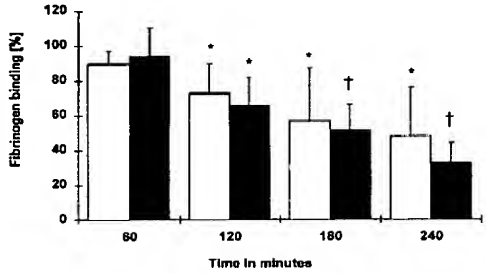

Figure 5. Fibrinogen binding to the GP IIb/IIIa receptor after activation with ADP (FC, 2 μmol/L) as a percentage of baseline value at 60, 120, 180, and 240 minutes. NO was administered in vitro at 100 (open bars) and 884 (solid bars) ppm NO (mean±SEM; *$P<.05$, †$P<.01$ vs t_0).

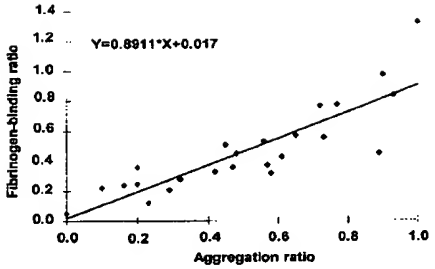

Figure 6. Correlation between change in platelet aggregation (aggregation ratio) and change in fibrinogen binding to GP IIb/IIIa receptor (fibrinogen-binding ratio) during administration of 884 ppm NO in vitro (statistical analysis: Pearson test, $R=.86$, $P<.0001$).

binding to the GP IIb/IIIa receptor of human platelets in vitro in a dose-dependent manner. These results could be substantiated in a clinical situation: besides prolonging the in vitro bleeding time, NO inhalation in patients with ARDS led to an inhibition of platelet aggregation, P-selectin expression, and fibrinogen binding.

In addition to their vasodilatory properties, systemically administered NO-releasing compounds have been shown to inhibit platelet function in vitro and in vivo,[1,3,24] and their antithrombotic effect is proposed to be beneficial.[25] The inhibition of platelet function may be explained by an activation of the soluble guanylate cyclase in the platelet cytosol, leading to an increase in cGMP levels.[17] Increased cGMP levels in platelets can induce a decrease in intracellular calcium ion concentration, which may contribute to an inhibition of fibrinogen binding to the GP IIb/IIIa receptor on the surface of the platelet membrane, which mediates platelet aggregation.[2,3,17,26] Furthermore, an increase in intracellular cGMP levels at the early stage of aggregation has been proposed, which could be further enhanced by relatively low concentrations of NO and finally lead to platelet disaggregation.[14] In the present study, we were not able to determine intracellular cGMP levels. However, NO therapy in patients with ARDS led to a significant increase in plasma cGMP levels, which have also been demonstrated to reflect NO-induced guanylate cyclase activation in vivo.[27-29]

The inhibition of platelet aggregation was reported to be dependent on the NO concentration in vitro and in healthy volunteers.[12-17] Moreover, the antiplatelet effect was persistent despite a hemodynamic tolerance during nitroglycerin therapy in pigs.[30] Conversely, bleeding time was shortened after systemic inhibition of NO production.[31] The results of the present study are in accordance with these findings, demonstrating a dose-dependent inhibition of both ADP- and collagen-induced platelet aggregation during NO administration in vitro. In comparison, in another study, 200 and 400 ppm inhaled NO were found to significantly inhibit ADP-induced maximal platelet aggregation.[32] When collagen was used to induce platelet aggregation, however, this inhibition was observed later and to a lesser extent, as also reported by other investigators.[10,33,34] This may be explained by the relatively high collagen concentration that was used in the aggregation studies. Lower concentrations of collagen or the use of epinephrine, which led to good results in the in vitro

TABLE 1. ADP- and Collagen-Induced Platelet Aggregation and ADP-Activated P-Selectin Expression and Fibrinogen Binding to the GP IIb/IIIa Receptor as a Percentage of Baseline Values at 60, 120, 180, and 240 Minutes in Older Volunteers (55 to 65 Years Old)

	Time, min			
	60	120	180	240
ADP-induced platelet aggregation (FC, 5 μmol/L), %				
100 ppm	93±8	78±9*	63±9†	37±8†
884 ppm	46±8†	39±11‡	22±8‡	16±7‡
Collagen-induced platelet aggregation (FC, 0.19 mg/mL), %				
100 ppm	84±14	76±14	60±10*	36±2†
884 ppm	77±4*	54±9†	45±6‡	22±11‡
P-selectin expression (FC, 2 μmol/L), %				
100 ppm	70±9	78±7	72±4	47±5*
884 ppm	70±14	64±12*	58±11†	39±10‡
Fibrinogen binding (FC, 2 μmol/L), %				
100 ppm	72±27	68±17	60±14	39±4*
884 ppm	70±17	35±8†	38±9†	30±9†

NO was administered in vitro at 100 and 884 ppm.
Values are mean±SEM.
*$P<.05$, †$P<.01$, ‡$P<.001$ vs baseline.

bleeding time measurements, might have revealed a comparable platelet inhibitory effect.

In the present study, inhibition of ADP-induced platelet aggregation and prolongation of in vitro bleeding time were also observed during inhalation of 10 ppm NO in vivo. A prolongation of bleeding time during NO administration was observed in rabbits,[8] whereas in rats, no change in bleeding time was observed at 80 ppm NO.[35] In healthy volunteers, 30 ppm inhaled NO also prolonged bleeding time[9] without an effect on filter aggregometry findings.[28] Other investigators observed no effect of inhaled NO either on β-thromboglobulin or on thromboxane B_2 levels.[36] Recently, it was shown that 3 to 30 ppm inhaled NO inhibits platelet aggregation without prolonging bleeding time in patients with ARDS.[10] In addition, platelet activation was abolished during NO administration in pigs after acute pulmonary embolism.[11] In accordance with our findings, most of these studies demonstrated inhaled NO to have an inhibitory effect on platelet function. Different findings with regard to bleeding time and platelet aggregation may be explained by the different methods and study populations that were used[10,11,18,23,28] and are therefore not necessarily contradictory.

In contrast to the in vitro findings, we cannot say whether the inhibition of platelet function by inhaled NO is also dose dependent in vivo, because only one NO concentration was administered. The maximal inhibition of platelet aggregation was already achieved at 3 ppm NO during inhalation of 1 to 100 ppm NO in a comparable study population.[10] In healthy volunteers inhaling 30 ppm NO, no further prolongation of bleeding time was observed at 80 ppm.[28] In comparison with these studies, no definitive dose-related effect of inhaled NO on platelet function could be found in animals,[8,11] and further studies are necessary to investigate a dose relation in a larger study population.

In activated platelets, P-selectin is translocated to the platelet surface from α-granules.[20] Inhibition of NO synthesis led to an increase in P-selectin expression.[37] Furthermore, the

TABLE 2. ADP-Induced Maximal Platelet Aggregation (FC=5 μmol/L), ADP-Stimulated P-Selectin Expression and Fibrinogen Binding (FC=2 μmol/L) as a Percentage of Baseline Values 180 and 240 Minutes After the Beginning of NO Administration (50 ppm) In Vitro

	Time, min		
	180	240	*P*
Platelet aggregation (FC, 5 μmol/L), %	87±10	63±11	<.05
P-selectin expression (FC, 2 μmol/L), %	92±21	85±19	<.07
Fibrinogen binding (FC, 2 μmol/L), %	73±21	64±9	<.05

Values are mean±SEM.

TABLE 3. Platelet Count, White Blood Cell Count, Plasma cGMP, In Vitro Bleeding Time, ADP-Induced Platelet Aggregation, Basal and ADP-Stimulated P-Selectin Expression, and ADP-Stimulated Fibrinogen Binding in Patients With ARDS Before (0) and 120 Minutes After Start of Administration of 10 ppm Inhaled NO

	Time, min	
	0	120
Platelet count, cells/nL	171±26	176±28
White blood cell count, cells/nL	15.4±1.3	15.2±1.6
cGMP, ng/mL	4.7±1.1	7.7±1.4*
In vitro bleeding time, s	71±11	99±13*
Platelet aggregation (FC, 5 μmol/L), %	26±2	19±3*
Basal P-selectin expression (FC, 2 μmol/L), %	10.1±2.2	6.7±1.7*
ADP-stimulated P-selectin expression (FC, 2 μmol/L), %	57±7	43±6*
Fibrinogen binding (FC, 2 μmol/L), %	30±8	19±7*

Values are mean±SEM.
*$P<.05$ vs baseline.

administration of NO-releasing compounds inhibited P-selectin expression in vitro[16,26,38] and in several clinical conditions.[21,38,39] In keeping with the data from the aggregation study, dose-dependent inhibition of P-selectin expression in ADP-activated platelets was observed in the present study during NO administration in vitro. In patients with ARDS, basal and ADP-activated P-selectin expression were also inhibited during NO inhalation, reflecting its platelet inhibitory effect. P-selectin also mediates leukocyte and endothelial interaction, which was significantly affected by NO in vivo.[40] In the present study, the platelet and white blood cell counts remained stable during NO administration, most likely excluding a relevant leukocyte or platelet sequestration.[41,42]

A decrease in platelet fibrinogen binding was observed after administration of various NO-releasing compounds in vitro and in vivo.[26,43,44] Furthermore, the increase in GP IIb/IIIa receptor expression was abolished during administration of *S*-nitrosoglutathione and nitroglycerin in patients with unstable angina and acute myocardial infarction[21] and during percutaneous transluminal coronary angioplasty.[38] The results of the present study are comparable to these findings, because the binding of fibrinogen to the GP IIb/IIIa receptor was inhibited in vitro and during NO inhalation in patients with ARDS. As with the results of the aggregation studies, this inhibition was dose dependent in vitro. Moreover, in the present study, a significant correlation between aggregation ratio and fibrinogen-binding ratio was found, suggesting that the prolongation of the in vitro bleeding time and the inhibition of platelet aggregation are most likely due to the inhibition of fibrinogen binding to the platelet surface GP IIb/IIIa, which is required for platelet adhesion and aggregation.[2,3] Because we were not able to measure the amounts of GP IIb/IIIa receptors on platelets,[45] we cannot say whether inhaled NO influences the structure or the number of expressed GP IIb/IIIa receptors or both. Nevertheless, recent studies suggest that NO may inhibit the number of expressed GP IIb/IIIa receptors in vitro and in vivo.[21,38,46,47] Thus, the expression of GP IIb/IIIa receptors may also be influenced by inhaled NO, which may contribute, at least in part, to the inhibition of fibrinogen binding, as demonstrated in the present study.

Although the in vitro model seems to be useful because it was possible to obtain mechanical platelet activation by means of gas bubbles[48] and a filter membrane was used to imitate the alveolar membrane, the in vitro findings cannot be completely extrapolated to the clinical setting. In the present study, relatively high concentrations of NO were used in vitro. Nevertheless, our results seem to be relevant, because a significant inhibition of platelet aggregation and fibrinogen binding was already observed at 50 and 100 ppm, concentrations that have been used in patients.[7,10] Furthermore, PRP was used, whereas in the lung, NO comes into contact with hemoglobin, which is known to inhibit its effects.[5,7] On the other hand, the inhibitory effect on platelet function may be more pronounced in the pulmonary vessels, where inhaled NO may improve the microcirculation, because NO inhalation decreased platelet sequestration in the lungs during extracorporeal circulation in pigs.[41] In the present study, however, the inhibition of platelet aggregation and fibrinogen binding in vivo supports the in vitro findings.

In conclusion, the results from the present study provide new evidence of an inhibitory effect of inhaled NO on platelet function. In fact, the inhibition of platelet adhesion and aggregation seems to be mediated via an inhibition of fibrinogen binding to the platelet membrane. The antiaggregatory effect could be beneficial: in a canine model of platelet-mediated reocclusion after thrombolysis, inhaled NO improved the coronary artery patency ratio,[32] and platelet activation was inhibited after pulmonary embolism in pigs.[11]

Because of its positive effect on hemodynamics and its antiplatelet action, the administration of inhaled NO in critically ill patients may be a beneficial and attractive adjunct to anticoagulation therapy. The present study provides a rational basis for investigating the efficacy of inhaled NO as an antiplatelet agent in further clinical studies.

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